AD-A268 902

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CONTRACT NO: DAMD17-90-C-0064

TITLE: GENETIC VARIATION OF HIV: VIRAL LOAD AND GENOTYPIC DIVERSITY IN RELATION TO VIRAL PATHOGENESIS AND TREATMENT

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REPORT DATE: July 20, 1993

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and
Development Command, Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
20 July 1993

3. REPORT TYPE AND DATES COVERED
Final Report (6-22-90 - 6-21-93)

4. TITLE AND SUBTITLE
Genetic Variation of HIV: Viral Load and Genotypic Diversity in Relation to Viral Pathogenesis and Treatment

5. FUNDING NUMBERS
Contract No.
DAMD17-90-C-0064

63105A
3M263105870.AA.023
WUDA335426

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Elucidation of Human Immunodeficiency Virus (HIV) pathogenesis and development of effective antiviral treatments and vaccines are priorities of the Army Retrovirus Program which require a thorough understanding of viral replication patterns, viral load (burden), and the molecular details of viral variation occurring in natural infection. Previously, little information existed concerning HIV-1 and HIV-2 replication and variation *in vivo* except for inferences drawn from studies of virus replication in cell culture, determinations of circulating p24 antigenemia, and genetic analysis of laboratory isolates of HIV. In our studies, we employed novel PCR (polymerase chain reaction), recombinant DNA, and virus culture approaches to study viral replication, burden, and variation in HIV infected individuals and in subjects receiving antiviral treatment. Specific aims were to:

1. Develop quantitative PCR-based approaches for measuring HIV-1 and HIV-2 DNA and RNA in blood and bone marrow mononuclear cells (PBMC/BMMC) and in plasma.

14. SUBJECT TERMS
HIV-1; HIV-2; Viral Replication; Viral Pathogenesis; Viral Quantitation; Vaccines; Genetic Variation; Zoonosis; PCR; RAD I

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Block 13 (cont)

2. Determine the relation between quantitative HIV-1 DNA and RNA levels determined by PCR and titers of cell-free infectious HIV-1 virus cultured from plasma.
3. Determine the relation between HIV-1 DNA and RNA levels in blood and other surrogate markers of HIV-1 infection (plasma p24 antigen and anti-p24 antibody levels, CD4 lymphocyte counts, β-2-microglobulin levels) currently utilized to monitor treatment efficacy.
4. Determine prospectively the relation between quantitative HIV-1 DNA and RNA levels as determined by PCR and disease stage, progression, prognosis, and response to therapy in HIV-1 infected individuals.
5. Utilize PCR amplification of HIV-1 DNA and RNA from uncultured tissues and also direct lambda phage cloning (without PCR amplification) from uncultured tissues to examine the molecular characteristics of HIV variation *in vivo* as a function of disease stage, time, and tissue type.
6. Examine the biological consequences of HIV-1 genotypic variation *in vivo* by analysis of transfected proviruses and provirus gene constructs derived from cultured and uncultured tissue specimens.
7. Utilize PCR amplification and "universal" (consensus) oligonucleotide primer sequences as an approach to identify, clone, and characterize divergent or novel human retroviruses in high risk populations.

We have addressed each of these seven specific aims. Our studies show for the first time: (i) the dynamic nature and extent of changes in viral replication and viral load that occur during natural infection from acute (CDC stage I) to chronic (CDC stages II-IV) disease as measured by infectious plasma viremia and direct PCR-based measures of total plasma viremia; (ii) substantial changes in genotypic complexity (quasispecies) occurring with sexual transmission of HIV-1 and the subsequent establishment of viral persistence by PCR-based sequencing of uncultured PBMC DNA; (iii) rapid changes in virus burden and the development of genetic and biologic resistance in response to nucleoside and non-nucleoside RT inhibitor therapy *in vivo*; (iv) genetic and biologic characteristics of full-length HIV-1 proviruses cloned directly from *cultured* and *uncultured* human tissue; (v) the identification by PCR amplification of HIV-2 viruses in humans that are phylogenetically indistinguishable from SIV_{SM} in sooty mangabeys, thereby providing direct evidence for HIV-2 as a zoonotic infection of man; and (vi) the presence of high ratios (average 60,000:1) of defective (non-culturable) to infectious virus in plasma throughout all stages of infection. These findings and their implications to HIV pathogenesis, treatment and prevention are described in the body of this report and in 17 published manuscripts and 35 published abstracts (Appendix B).

FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR56.

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"Genetic Variation of HIV: Viral Load and
Genotypic Diversity in Relation to
Viral Pathogenesis and Treatment"

Introduction

Infection with human immunodeficiency virus type 1 (HIV-1) causes a chronic progressive illness characterized by deterioration in immune and neurologic function and frequent abnormalities in other organ systems including hematologic, pulmonary, cardiac, renal, and gastrointestinal. Direct and indirect roles for HIV-1 have been postulated to explain the diverse clinical sequelae resulting from viral infection, but even the progressive loss of $CD4^+$ lymphocytes leading to the acquired immunodeficiency syndrome (AIDS) has not been explained mechanistically (1,2). HIV-1 is classified as a lentivirus because of its characteristic genomic organization, nontransforming biological properties, and slowly (lenti-) developing clinical sequelae that are analogous to other lentiviruses such as visna virus, equine infectious anemia virus (EIAV), and simian immunodeficiency virus (SIV) which infect different animal species (3,4). For these other lentiviruses, there is compelling evidence for the importance of viral replication patterns being critical determinants of viral pathogenicity and natural history. For EIAV and SIV in particular, high level viral replication leading to high titer plasma viremia has been directly associated with efficient transmission and disease induction (5,6).

Relatively little is known about replication patterns and viral burden of HIV-1 in vivo and their relation to disease pathogenesis. Recent studies have shown that both $CD4^+$ lymphocytes in blood and monocyte-derived cell types in brain serve as primary reservoirs for HIV-1 and that the proportion of virally infected cells and the abundance of viral DNA increases with disease

progression (7-10). Circulating HIV p24 antigen, a marker of HIV-1 replication, has been shown to occur transiently during acute HIV-1 infection and to reappear in later stages of infection coincident with a decline in $CD4^+$ lymphocytes (11-13). Cell-free infectious virus in plasma, another indicator of HIV-1 replication, was first demonstrated by Zagury and co-workers (14) followed by other reports of viremia in association with p24 antigenemia in early and late infection (15-20). A major objective of our studies was to better define the natural history of HIV infection by obtaining systematic and quantitative data for HIV-1 plasma viremia and its relation to p24 antigenemia, anti-p24 antibody, clinical stage, likelihood of disease progression, and response to antiviral therapy. Included in this objective was the development of new PCR (polymerase chain reaction) based approaches for more accurately quantifying HIV-1 nucleic acid in blood and plasma. A second major objective of the project was to characterize the genotypic and biologic evolution of virus *in vivo* throughout all stages of infection and in response to therapy. A third major objective was to develop PCR based techniques using generic (universal) oligonucleotide primers in an attempt to identify novel HIV-1/HIV-2 related immunodeficiency viruses. We have addressed each of these objectives. In this Final Report, the results of these studies and our interpretations are described.

Body (Results)

1. Quantitation of Infectious Virus in Patients with Chronic HIV-1 Infection.

Sixty-eight HIV-1 infected adults, 14 HIV-1 infected or exposed children, and 20 uninfected normal donors were enrolled in our studies of plasma viremia and viral pathogenesis. The clinical protocol was approved by the Human Use Committee of the University of Alabama at Birmingham (UAB) Institutional Review Board. Patients at all stages of clinical disease who attended the UAB

AIDS Outpatient Center and The Children's Hospital of Alabama were identified and recruited to participate in the study. All volunteers underwent detailed clinical and laboratory evaluations including lymphocyte subset analyses and were staged according to the Centers for Disease Control (CDC) classification system (21,22). Among the adult patients, 19 were asymptomatic (CDC stage II), 34 were classified as early AIDS-related complex (ARC; CDC stage III/IVC2), and 15 patients had AIDS (CDC stage IVA/B/C1/D). Among the 14 children, 5 were exposed to HIV-1 in utero but exhibited no clinical or laboratory evidence of infection (CDC stage P0) based on negative peripheral blood lymphocyte cultures, undetectable serum p24 Ag, decreasing intensity of reactivity of enzyme linked immunoabsorbent assay (ELISA) and Western blot for HIV-1 antibodies over time, and absence of polymerase chain reaction (PCR) amplification of HIV-1 DNA. The other 9 children, 5 of whom were exposed to virus in utero and the remainder by transfusion of blood or clotting factors, had established HIV-1 infection as determined by positive HIV-1 lymphocyte cultures and presence of anti-HIV-1 antibody in children $\geq$ 15 months of age. Three of the HIV-1 infected children had AIDS (P2B, C, D1, D2, or E1), five had clinical findings indicative of symptomatic HIV-1 infection (P2A, D3, E2, F, or IVC2), and one had asymptomatic infection with normal immune function (P1A).

For the entire group of adults and children, the CD4 counts ranged from 3 cells/mm^3 to 2848 cells/mm^3 and the CD4:CD8 ratios from 0.01 to 4.30. All but 7 patients were studied before the institution of antiviral therapy, and 22 patients were studied both before and after zidovudine treatment. One patient had taken dithiocarb (DTC) and another patient had taken hypericin. On the day of clinical evaluation, 60 ml of peripheral blood from adults and 3 ml from infants and children were obtained in sterile acetate citrate dextran (ACD) vacutainers via antecubital venipuncture. The blood was transported to the laboratory at ambient temperatures and within two

hours was centrifuged at 675 g x 15 minutes to separate plasma and cellular fractions. The collected plasma fraction was centrifuged at 1000 g x 15 minutes to remove residual cellular and platelet components. It was then passed through a sterile 1.2 μm nonabsorbing filter that had been prewetted with RPMI containing 15% heat inactivated fetal calf serum (FCS) to insure the removal of any residual cells or platelets. Filtered plasma was then serially diluted from 10^0 to 10^{-8} with RPMI-1640 containing 15% FCS. One milliliter aliquots of each plasma dilution (10^0 to 10^{-8}) were then combined in 12 well plates with 2 x 10^6 type O normal donor peripheral blood mononuclear cells (PBMC) (total volume 2 ml) which had been stimulated for 48-72 hours with phytohemagglutinin (PHA; 2 μg/ml), washed, and resuspended in RPMI-1640 containing IL-2 (30 units/ml), gentamicin (0.1 mg/ml), L-glutamine (2 mM), and 15% FCS. Cultures were maintained for five weeks with 1:3 cell splits performed weekly. Supernatants were tested weekly for HIV-1 p24 antigen in a solid phase sandwich-type enzyme linked immunosorbent assay (Abbott Laboratories, Chicago, IL) beginning on days 3 to 7. Positive cultures were defined as those which achieved absorbance values greater than 2 standard deviations above normal control samples (greater than 30 pg p24 antigen per ml) and had rising levels of HIV-1 p24 antigen reflecting active viral replication. Selected cultures were further characterized by reverse transcriptase assay and PCR analysis and were amplified and frozen for subsequent analyses. The final endpoint plasma titer represents the highest plasma dilution during the five week culture period yielding a positive result. Uncultured and unfiltered plasma samples from each study subject were frozen at -70°C and analyzed for quantitative p24 antigen levels as described elsewhere (20). Both plasma and tissue culture supernatant samples were treated with 0.5% Triton X-100 in order to disrupt virions prior to incubation with the primary solid phase anti-HIV-1 antibody. In addition, patient samples were also analyzed for p24 antibody levels using a new enzyme immunoassay developed at Abbott

Laboratories. In this assay, recombinant p24 antigen is used on the solid phase as well as conjugated to horseradish peroxidase to specifically capture p24 antibodies from plasma or serum. Serial five-fold dilutions of the specimen permits determination of the endpoint titer which is the greatest dilution at which p24 antibodies are still reproducibly detected. The endpoint titer is a function of the amount of antibody present in the specimen and the avidity of the p24 antibody population, this being a measure of the overall p24 antigen binding strength. Statistical analyses were performed by chi square analysis.

A total of 68 infected adults and 14 children were evaluated 161 times during the course of this study. Tables 1 and 2 summarize the initial plasma culture results for each subject with CDC stage II/P1 disease or greater in relation to other clinical characteristics including disease stage, $CD4^+$ lymphocyte counts, plasma HIV-1 p24 antigen and antibody levels, and concurrent antiretroviral treatment, if any. In the adult group, 14/15 patients with AIDS (CDC stage IVA/B/C1/D) were viremic compared with 4/53 patients with less advanced illness (CDC stage II/III/IVC2; $\underline{P} < 0.001$). In the pediatric group, 5 of 9 children with established HIV-1 infection were plasma viremic. Interestingly, this included all 5 children who had been infected with HIV-1 in the perinatal period and none of the 4 children infected after the age of 3 months as a result of blood product transfusion. None of five children (stage P0) who were exposed to HIV-1 in utero but who remained virus negative based on repeated lymphocyte cultures and absence of viral specific PCR amplification of viral DNA nor any of 20 additional HIV-1 seronegative control subjects included as blinded controls.

Levels of plasma viremia were determined for all subjects and ranged from 10^1 to 10^5 TCID (tissue culture infectious doses) per milliliter of plasma. The mean geometric endpoint titer for HIV-1 viremia in adults with $CD4^+$ lymphocyte counts of $>400/mm^3$, $200\text{-}400mm^3$, and $<200/mm^3$

were 0, $10^{0.5}$, and $10^{2.8}$, respectively. Of 15 patients with CDC defined AIDS, 7 had virus levels between 10^3 and 10^6 TCID/ml. For perinatally infected children, the geometric mean titer was $10^{2.6}$ TCID/ml plasma with a range of 10^1 to 10^3 TCID/ml. For the 24 viremic patients studied, plasma cultures became detectably positive at one or more dilutions by day 6 on average (range 3 to 14 days) while the time required for the final endpoint plasma culture to become positive averaged 12 days (range of 4 to 39 days). In 21 out of 23 viremic subjects, culture endpoint was reached by day 21. In all instances but one, all cultures corresponding to plasma dilutions less than the endpoint titer were also positive for virus (e.g., plasma dilutions 10^0 to 10^{-4} and 10^0 to 10^{-7} were positive along with the 10^{-5} and 10^{-8} endpoint cultures of patients DODO-0116 and JOJI-0070, Table 1).

Experiments were also performed to determine the consistency of plasma viral titers in untreated patients over time, the effect of normal lymphocyte donor selection on viremia titers, and the intra-experiment reproducibility of the assay. Twelve subjects who did not receive antiviral therapy were studied on more than one occasion over a 1-7 month period. All 5 individuals who were initially viremic remained so, and similarly, all 7 individuals who were initially not viremic remained without detectable virus in their plasma. Of the 5 viremic subjects who did not receive antiviral therapy (BIJA-0205, DODO-0116, SMDO-0157, DEDA-0006, and WHRO-C002), virus titers varied by 2 log dilutions or less (Table 3). Plasma cultures for two subjects (BIJA-0205 and NAPH-0073) were each established using cells from three different normal donors (D2,D3, D4 and D15, 16, 17, respectively) and from single normal donors in triplicate (D4a, b, c and D17 a, b, c, respectively), and the titers of HIV-1 measured varied by 1 log dilution or less (Table 3).

A striking inverse correlation was found between the presence of viremia and $CD4^+$ lymphocyte counts in adult patients (Figure 1). Fifteen of 16 subjects with <200 $CD4^+$ cells/mm^3

and 3 of 17 subjects with 200-400 $CD4^+$ cells/mm^3 were viremic as opposed to 0 of 35 subjects with $CD4^+$ lymphocyte counts > 400 cells/mm^3. In contrast to these results in adults, all 5 children who acquired HIV-1 infection perinatally were viremic regardless of their $CD4^+$ lymphocyte counts which ranged from 42 to 2848 cells/mm^3 (Table 2). Children infected by transfusion of blood products at older ages were similar to adult patients with none of them having plasma viremia.

Of 77 HIV-1 infected subjects, 30 had detectable HIV-1 p24 antigen in plasma. There was a positive correlation between level of p24 antigenemia and HIV-1 viremia ($r = 0.60$; $\underline{P} < 0.001$; Figure 2), although in both adults and children there was substantial discordance between the two measures of viral replication. For example, of 18 adults and 5 children who were viremic, 4 (22%) and 2 (40%), respectively, lacked detectable p24 antigen in their plasma. Conversely, among 50 adults and 4 children who were not viremic, 10 (22%) and 3 (75%), respectively, were p24 antigenemic. Of 16 adults with $CD4^+$ counts less than 200/mm^3, 15 (94%) were viremic and 12 (75%) were antigenemic. Of 17 adults with $CD4^+$ counts between 200 and 400/mm^3, 3 (18%) were viremic and 6 (35%) were antigenemic. Of 35 adults with $CD4^+$ counts greater than 400/mm^3, none were viremic and 6 (17%) were antigenemic. Of 15 patients with CDC defined AIDS, 14 had HIV-1 plasma viremia and 11 HIV-1 p24 antigenemia. The mean antigen levels in viremic and nonviremic groups of patients were 130 pg/ml and 7 pg/ml, respectively ($\underline{P} < 0.001$).

Anti-p24 antibody assays were performed on all but four HIV-1 infected study subjects. Fifty-four out of 73 individuals studied had detectable anti-p24 antibodies. Twenty-nine of 36 (81%) subjects with > 400 $CD4^+$ cells/mm^3, 17 of 19 (89%) subjects with 200-400 $CD4^+$ cells/mm^3, and 8 of 18 (44%) subjects with <200 $CD4^+$ cells/mm^3 had anti-p24 antibodies. Of 15 subjects with CDC defined AIDS, 14 were viremic and 8 had anti-p24 antibodies. Forty-three of 52 (83%) nonviremic subjects had anti-p24 antibodies as opposed to 11 of 21 (52%) viremic subjects. The

mean titers of anti-p24 antibodies in nonviremic versus viremic subjects were 17,756 and 62, respectively ($\underline{P} < 0.001$).

Nineteen adults and 3 children were evaluated before and after initiation of therapy with zidovudine (dosages ranging from 300 mg/d to 1200 mg/d). Among the adults, 13/19 had negative plasma cultures pretreatment and all 13 remained without plasma viremia while on zidovudine (mean follow-up 18.5 weeks; range 8-32 weeks). Among the 6 adults and 2 children who were viremic on initial evaluation (pretreatment), all 8 exhibited a decrease in plasma viremia titers by 10 to 10^6 fold (Figure 3) with a geometric mean pretreatment titer of $10^{2.9}$ which decreased to $10^{0.6}$ while on zidovudine (mean follow-up 18.5 weeks, range 15 to 40 weeks). Five of the eight patients became plasma culture negative while receiving zidovudine therapy. In contrast, four adults who were viremic on initial evaluation and did not receive antiviral therapy remained viremic on subsequent assessments with an initial geometric mean titer of $10^{4.0}$ versus a mean follow-up titer of $10^{3.5}$ TCID (mean duration of follow-up 18 weeks; range 1 to 32 weeks). HIV-1 p24 antigen was detectable in 10 patients (8 adults and 2 children) before initiation of zidovudine therapy and decreased in 8 of them during therapy with mean values falling from 166 pg/ml to 117 pg/ml (mean follow-up 21 weeks, range 6-40 weeks).

2. <u>Quantitation of Infectious Virus in Patients with Acute HIV-1 Infection.</u>

We also determined the level of cell-free infectious virus in plasma samples in four subjects with acute HIV-1 infection. Acute HIV-1 infection (CDC stage I) represents a dynamic period during which high level viral replication and widespread viral dissemination occur. These virologic events frequently occur in association with severe clinical symptoms (Table 4) and signs of immune activation and are followed by prompt resolution of plasma viremia, antigenemia, and clinical symptoms. In our studies, we determined the titers and biological properties of infectious HIV-1

in plasma during primary, symptomatic infection in three patients and studied the relation of these factors to p24 antigenemia, seroconversion, and clinical course. We also performed molecular analyses of HIV-1 proviruses from an acutely infected person and his sexual partner, and we documented that sexual transmission and the resulting acute, symptomatic infection are associated with high-level expression of replication-competent, cytopathic virus. Finally, we employed polymerase chain reaction (PCR) amplification of HIV-1 DNA sequences from uncultured peripheral blood mononuclear cells (PBMC) to show that acute HIV-1 infection is associated with high level replication of a relatively homogeneous population of virus which then evolves over time into the quasispecies mixture of viruses characteristic of established HIV-1 infection.

Figure 4 demonstrates the seroconversion profiles of four acutely infected individuals. Patients #1-#3 correspond to the same patients whose antigenemia and viremia titers are profiled in Figure 5. In all patients, the peak plasma virus titers were observed during the earliest, most symptomatic phase of acute illness, approximately 6 to 15 days after the onset of symptoms and at a time when all serological assays for HIV-1 antibodies were negative. HIV-1 specific seroreactivity as determined by ELISA first became positive (ratio of sample/cutoff for normal control > 1.0) between 11 and 20 days after the onset of symptoms and specific reactivity to HIV-1 *gag* (p24) and *env* (gp160) proteins on Western immunoblot was first detectable at 9 to 24 days. Broader reactivity to additional viral proteins followed, along with an increase in total antibody response reflected in rising absorption titers on ELISA over the course of the study.

HIV-1 TCID titers in plasma ranged from 10^1 to 10^3 TCID per milliliter initially. In a fourth patient (SUMA), peak titers were 10^4 TCID/ml. In three of the patients, plasma virus titers decreased rapidly within 8 to 14 days at the same time that a strong HIV-1 antibody response appeared. These patients had no detectable plasma viremia 27 and 14 days after the onset of

symptoms. Patient 2, (INME), on the other hand, did not have complete resolution of plasma viremia until 75 days after onset of symptoms. Of the three patients studied, this patient had the latest appearance of serologic reactivity to *gag* and *env* proteins. Once plasma viremia resolved, it was not again detected in any of the four patients in cultures performed over the next one to six months. In all four patients, levels of HIV-1 p24 antigen closely paralleled the level of virus in plasma ($r = 0.73$, $p < 0.0001$), and both were inversely correlated with anti-HIV-1 ELISA reactivity ($r = -0.72$ p24, $r = -0.73$ viremia, $p < 0.001$).

HIV-1 was also cultured from PBMCs obtained from patients at the time of initial presentation and subsequently. Virus isolated from the plasma and PBMCs of all three patients with primary infection replicated to high levels and formed syncytia in PBMC cultures, and isolates established productive infections in H9 and Hut 78 after cell-free transmission. Sequential virus isolates from Patient 1 obtained on days 15, 51, and 86, as well as genetically defined virus strains derived by molecular cloning and transfection of HIV-1 proviruses from Patient 1 obtained on day 15, were all highly cytopathic and exhibited replicative and fusogenic properties ($>10^4$ $TCID_{50}$/ml; $>10^4$ cpm/ml RT activity; $>10^4$pg/ml p24 antigen in culture supernatants) equivalent to the most cytopathic strains of HIV-1 previously described.

The genetic composition of HIV-1 virus that is transmitted sexually is unknown. Therefore, we used PCR amplification of viral DNA from *uncultured* PBMC to characterize envelope V3 loop sequences from an individual with AIDS (patient RIER) who transmitted virus to patient # 1 (WEAU; shown in Figure 4 and 5). A summary of that analysis is depicted in Figure 6. In patient RIER, sequence diversity characteristic of established HIV-1 infection was found. The predominant viral species present in RIER lymphocytes was the only viral form transmitted to the recipient WEAU. As shown, sequence analysis of 24 viral clones from 5/30/91, 20 clones from 6/21/90,

and 22 clones from 1/18/91 demonstrated that acute viral infection is associated with high level replication of a relatively homogeneous population of viruses and that over time virus complexity broadens. Interestingly, few changes evolved and persisted within the V3 loop of HIV-1 in this patient over an eight month period.

Analysis of the genetic and biologic characteristics of the viral strains present in patients RIER and WEAU provided certain insights into the pathogenesis of HIV-1 disease. The restriction-enzyme cleavage patterns of the predominant viruses from both patients were identical, but they differed from those of isolates from unrelated subjects in 48 to 68 percent of the sites mapped. Proof of transmission between these patients was obtained by sequence analysis of the viral envelope (Figure 6). Proviruses molecularly cloned from the initial viral culture of Patient 1 (WEAU) were shown, after transfection into Cos cells and cell-free passage onto H9 cells, to be fully replication-competent and highly cytopathic. These data, along with the finding of high titer plasma viremia in most individuals with symptomatic primary infection, suggest that virus with high replicative potential may be commonly be transmitted by sexual routes in such individuals and accounts for their clinical symptoms and rapid seroconversion usually within one to three months. Other persons may be exposed to smaller amounts of virus, to less virulent viral strains, or even to defective virus, which could explain the reports of prolonged virus-positive, antibody-negative periods in persons with subclinical primary infection.

This study, and two other reports (19,20), were the first to examine in a systematic and quantitative fashion the role of plasma viremia in HIV-1 natural history and pathogenesis. Our report differs from the other reports by inclusion of children exposed to HIV-1 in utero and postpartum, the methodology for plasma virus cultivation, and the resultant data concerning the magnitude of viremia and its relation to CD4 lymphocyte levels and clinical stage in adult and

pediatric groups. The principal findings were that HIV-1 plasma titers reached levels as high as 10^6 TCID/ml, that viremia was closely associated with advanced disease and low $CD4^+$ lymphocyte counts in adults and older children but not in perinatally infected infants, and that treatment with zidovudine led to a decrease in plasma virus titers in most patients.

Whereas the overall geometric mean titers of plasma HIV-1 in AIDS patients in all three studies (19,20) were similar ($10^{2.5}$ to $10^{2.8}$ TCID/ml), maximum titers in our study (23,24) reached 10^6 TCID/ml with 4 of 14 viremic patients with AIDS having $\geq 10^4$ TCID/ml. In studies by Ho (19) and Coombs (20), maximum virus titers in plasma were between 2×10^4 and 5×10^4/ml with only 1 of 20 AIDS patients in one study having titers reaching 10^4/ml. Thus, our data indicate that a greater range of quantitative plasma viremia exists in HIV-1 infection and that a substantial proportion of AIDS patients has HIV-1 plasma titers equalling or exceeding 10^4/ml. Another difference between the results of this study and others is the proportion of HIV-1 infected individuals at earlier stages of infection who were found to be plasma viremic. All 54 HIV-1 infected individuals studied by Ho et al. (19) had cell free infectious virus in their plasma regardless of clinical stage or $CD4^+$ lymphocyte counts. Coombs, et al. (20) detected plasma viremia was detected in 75 of 92 patients with AIDS, in 32 of 71 patients with advanced ARC, and in 11 of 48 patients with asymptomatic infection. In our study, 15 of 16 AIDS patients were viremic whereas only 4 of 34 ARC patients and none of 19 asymptomatic individuals had detectable viremia. We also identified four adult subjects with acute HIV-1 infection (CDC stage I) and determined each of their cell-free plasma infectious HIV-1 titers to be 10^1 - 10^4 TCID/ml.

Methodological differences in procedures for establishing plasma cultures may explain the differences in results of between our study and others. Ho et al. (19) centrifuged the blood of HIV-1 infected subjects at high gravitational forces (3000 g) as opposed to our procedure (675 g)

to separate plasma from cells and they did not subsequently filter the plasma before cultivation. Lymphocyte-plasma cultures were then washed after 24 hours to remove patient plasma from the cultures so that no plasma was present for the duration of the 4 week culture period. Coombs et al. (20) initially diluted the blood of HIV-1 infected subjects 1:1 with saline prior to underlayering with lymphocyte separation medium and centrifugation at 800 g. The saline diluted plasma was then filtered prior to co-culture with PHA-stimulated normal donor lymphocytes in medium containing DEAE dextran. In our study, patient blood was centrifuged at low gravitational forces initially (675 g), and plasma was collected and centrifuged again at 1000 g prior to passage through a 1.2 μm filter to remove any residual cellular or platelet elements. Plasma dilutions were then added to PHA-stimulated PBMCs and cultured for 5 weeks without washing plasma from the cultures. It is thus possible that in the previously reported studies virus could be released from damaged cells in the preparation phase or be actually transmitted in platelet- or cell-associated form, whereas, in our procedure, low titer virus could be either lost by filtration or neutralized by the patients' minimally diluted plasma.

In contrast to the close correlations observed between plasma viremia, advanced disease, and low $CD4^+$ lymphocyte counts in adults, we found all 5 perinatally infected children to be viremic regardless of $CD4^+$ count, duration of infection, or clinical stage. For example, three of five children (HUJO-C005, MOBG-C004, GRAL-C015) with perinatal HIV-1 infection and plasma viremia had CD4-lymphocytes counts within the normal range for age-matched control subjects (see Table 2 legend) as opposed to eighteen viremic adults all of whom had CD4-lymphocyte counts less than 400/mm^3. The five viremic children also had much shorter durations of infection ranging from 6 weeks to 3 years and clinical stages ranging from asymptomatic (P1) to AIDS (P2B, C, D1, and D2). In contrast, viremic adults were uniformly symptomatic and infected for more than 3

years. These findings suggest that either adults are immunologically more capable than neonates of controlling HIV-1 replication for prolonged periods or the developing immune system of neonates supports greater viral replication than that of older children and adults. The ability of adults to clear the acute viremic phase of initial HIV-1 infection (23,24), the demonstration of protection from SIV induced disease in macaques and mangabeys by immunization or prior infection with attenuated viral strains (6), and the more rapid progression of HIV-1 disease in children as compared to adults (25) argues for an important role for the host immune system in suppressing viral replication.

We also examined the relationship between plasma p24 antigen and antibody levels, plasma virus titers, and clinical stage. Plasma viremia was highly predictive of AIDS (14 of 15 adults) and $CD4^+$ lymphocyte counts less than $200/mm^3$ (15 of 16 adults). In contrast, although p24 antigen and antibody levels were positively and negatively related to these clinical parameters, the correlations were weaker by comparison. Like previous workers (19,20), we were able to culture cell-free virus from a significant proportion of patients who were not antigenemic (6 of 24) or who possessed detectable levels of anti-p24 antibody (11 of 21). The strong association of infectious plasma HIV-1 titers with clinical stage and the weaker association of p24 antigen and antibody with either viremia or clinical stage suggest that quantitative plasma HIV-1 cultures may provide an important marker of efficacy in future trials of antiviral therapy. In support of this, we found all 8 viremic patients to have a decrease in mean plasma virus titers from $10^{2.9}$ to $10^{0.6}$ following treatment with zidovudine with 5 patients clearing plasma virus altogether. Ho et al. also studied 7 patients before and after initiation of zidovudine therapy and found a mean decrease in plasma viremia titers of 1600 TCID/ml (19). In further support of the role of zidovudine in reducing plasma viremia, the single AIDS patient in our study who was not viremic had received high-dose

(1200 mg/d) zidovudine for over one year at the time of study.

In summary, the results of this study provided direct virologic evidence for a model of HIV-1 natural history and pathogenesis (Figure 7). In adults, following acute HIV-1 infection by sexual or parenteral routes there is an early viremic phase (CDC stage I), followed by a prolonged period lasting years in which infectious viremia is generally low level or undetectable (CDC stages II and III), followed by a terminal phase of high level infectious viremia that is associated with profound immune deficiency (CDC stage IV). Children infected in utero or perinatally differ from adults in frequently exhibiting persistent infectious viremia and accelerated disease regardless of $CD4^+$ lymphocyte counts, duration of infection, or clinical stage, most likely as a result of a failure of the immune system to control viral replication. This model is consistent with clinical studies documenting accelerated clinical deterioration in HIV-1 infected neonates compared with adults (25), increased frequency of heterosexual viral transmission to spouses of hemophiliacs with advanced disease (26), and increased frequency of acute retroviral syndrome and progression to AIDS in recipients of HIV-1 infected blood products derived from individuals in advanced stages of illness (27). These data thus argue for the importance of the host immune system in controlling viral replication, and the need for early intervention with effective antiviral agents especially in perinatally acquired infection. They also suggest that certain individuals with high titers of cell-free infectious virus may be at increased risk for transmission of virus (28,29). Finally, the demonstration of high level HIV-1 viremia in vivo suggest that cell free HIV-1 virions, proteins, and antigen-antibody complexes may play a direct role in the natural history and pathogenesis of HIV-1 infection as occurs in infections by equine infectious anemia virus (5).

3. <u>Quantification of Total (Culturable and Non-Culturable) Plasma Virus by Competitive PCR in Acute and Chronic Infection.</u>

The natural history of HIV-1 infection is characterized by a variable clinical course, with the development of acquired immunodeficiency syndrome (AIDS) generally occurring after 7 to 11 years. A central paradox of HIV disease involves the progressive development of immunologic abnormalities, beginning during the early stages of infection when assays for circulating p24 antigen and culturable virus in peripheral blood suggest minimal or absent levels of viral replication (19,20,23,24). Recent studies (30) of HIV-1 DNA and RNA in clinical samples suggest that, as compared with peripheral blood cells, lymphoid tissue represents a preferred and continuous site of viral replication, although such studies have necessarily been limited by the relative inaccessibility of the tissue compartment, especially for repeated evaluation. Previous polymerase chain reaction (PCR) studies of HIV-1 RNA in plasma have generally been limited to qualitative or semiquantitative analyses (reviewed in 31,32). In standard PCR methods, the absolute amount of product generated does not always bear a consistent relation to the amount of target sequence present at initiation of the reaction, particularly for clinical specimens. Both the kinetics and efficiency of amplification of a target template are dependent on the starting abundance of that template and on the sequence match of the primers and target template and may also be affected by inhibitors present in the specimen (31,32). In PCR analysis of RNA samples, variable efficiencies in both the reverse transcription and amplification steps are potential sources of variability. For these reasons, comparison of the amount of specimen-derived PCR product to the amount of product from a separately amplified external control standard does not provide a rigorous basis for absolute quantitation. Normalization based on coamplification of a heterologous' internal "control" target sequence (such as β-globin or actin) does not optimally address this problem, owing to different template abundances and priming efficiencies for different primer-target combinations.

In thc quantitative competitive PCR (QC-PCR) method for RNA quantitation (31) a comoetitive RNA template matched to the target sequence of interest, but differing from it by virtue of an introduced internal deletion, is used in a competitive titration of the reverse transcription and PCR steps, providing stringent internal control. Increasing known copy numbers of competitive template are added to replicate portions of the test specimen, and quantitation is based on determination of the relative, not absolute, amounts of the differently sized amplified products derived from the wild-type and competitive templates, after electrophoretic separation. To increase the sensitivity and consistency of amplification of only virion-associated RNA, we pelleted virus from plasma by ultracentrifugation, used procedures intended to maximize recovery of extracted RNA, and targeted a highly conserved sequence in HIV-1 *gag* (31), using oligonucleotide primers that incorporate neutral inosine residues at the few positions of recognized variability.

Sixty-six consecutively enrolled HIV-1-infected subjects representing all stages of infection [Centers for Disease Control (CDC) stages I to IV] and ten HIV-1 seronegative healthy donors were evaluated for virion-associated HIV-1 RNA by QC-PCR. Infected subjects were also tested for culturable virus and for p24 antigen with both standard and immune complex dissociation (ICD) test procedures. For each subject, all virologic measurements were made with a single plasma sample, which was divided and frozen in replicate portions.

All patients were evaluated a the University of Alabama at Birmingham, where approval from the human subjects review board and informed consent were obtained. Blood specimens were collected in acid-citrate-dextrose and processed within 3 hours of phlebotomy. After centrifugation (200g for 15 min), plasma was collected and centrifuged again (1000 g for 15 min) to ensure cell-free specimens. Replicate portions of plasma were used immediately for virus culture or stored

at $\leq$ -70°C until further analysis. For extraction of virion-associated RNA, plasma samples were thawed and subjected to ultracentrifugation (Beckman type 70.1 rotor, 70,000 rpm, 1 hour) to pellet virions. Pellets were resuspended in solution [20mM tris (pH 7.5), 150 mM Na Cl, 2 mM EDTA], adjusted to contain 1 mg of proteinase K per milliliter and 0.1% SDS, and incubated at 37°C for 1 hour After repeated extraction with phenol:chloroform:isoamyl alcohol (24:24:1) followed by one extraction with chloroform, samples were adjusted to contain ~ 40μg of glycogen per milliliter (as carrier) and 10 ng of 7.5-kb synthetic RNA per milliliter (BRL, Bethesda, MD) (as carrier and to normalize total RNA content in the reverse transcription and PCR steps). RNA was then precipitated with ethanol at -20°C for 48 hours and pelleted by ultracentrifugation to maximize recovery. The RNA pellets were partially dried and then dissolved in 100 μl of sterile, ribonuclease-free water. Samples were stored at -70°C until subsequent analysis. The primers GAG04 (CATICTATTTGTTCITGAAGGGTACTAG) and GAG06 (GCITTIAGCCCIGAAGTIATACCCATG) have been described previously (31) and were designed to amplify an internal fragment of either 260 bp (from wild-type HIV-1 target sequences) or 180bp (from the PQP1Δ80 competitive template). To maintain equivalent and competitive priming efficiency with divergent sequences, primers incorporated inosine residues at the few positions where divergence from the conserved consensus sequence has been reported. For QC-PCR analysis, two plasmids were prepared, one containing the target sequence (PQP1) and the other containing the identical sequence except for an 80-bp internal deletion (pQP1Δ80), sufficient to allow the derived PCR products to be readily resolved by electrophoresis. In vitro RNA transcripts were prepared with commercially available kits. Final preparations in water were determined to be essentially free of degradation products by Northern (RNA) blot analysis and were quantified by measurement of absorbance at 260 nm. Portions were stored at -70°C until needed. PCR

reaction conditions and protocols were generally similar to those found in commercially available kits (Perkin-Elmer, Norwalk, CT). Each test sample was divided into eight replicate portions and analyzed in the presence of 0 to 50,000 copies per reaction of competitive template. The initial reaction was performed in a total volume of 30 μl and contained 5 μl of test RNA (corresponding to 5% or less of the total specimen), 5 μl of competing RNA preparation or water and 30 U of cloned Moloney virus reverse transcriptase (BRL, Bethesda, MD). One portion from each specimen was analyzed without reverse transcription and in the absence of competitive template. After 10 min at room temperature to allow partial extension and stabilization of random hexamer primers, conversion of RNA in to cDNA was allowed to continue for 30 min at 42°C. This reaction was then adjusted to contain primers and additional buffer in a total volume of 60 μl. Amplification was performed as described (31), with 45 cycles (94°C for 1 min, 50°C for 2 min, and 72°C for 1 min), followed by a final incubation at 5°C for 5 min. After amplification, approximately 7% of each reaction product mixture was separated by electrophoresis in composite 2% Synergel (Diversified Biotech, Newton Center, MA)--1% agarose (FMC Bioproducts, Rockport, ME) gels in 20 mM tris acetate (pH 7.8) and 1 mM EDTA. Gels were stained with ethidium bromide for visualization under ultraviolet illumination. Quantitation of fluorescence of both wild-type and competitive template product bands was performed on a Lynx 4000 molecular biology workstation with matched custom software (Applied Imaging, Santa Clara, CA). Competition equivalence points were determined by interpolation on plots of the logarithm of the calculated ratio of signal for the competitive template-derived product over the signal for the wild-type target sequence-derived product (corrected for molar ratio) versus the logarithm of the copy number of added competitive template (Fig. 1).

Endpoint dilution cultures of plasma for cell-free infectious virus were determined for fresh

specimens, generally in duplicate or quadruplicate. Plasma samples were not filtered (to eliminate the possibility of inadvertent loss of virus). Regular and ICD p24 antigen determinations were performed in duplicate with the Coulter Diagnostics kit assay, according to the manufacturer's recommendations (Coulter, Hialeah, FL). Analysis of variance, Duncan's multiple range test, and Tukey's Studentized range (HSD) test was calculated by the SAS statistical analysis software package (SAS Institute, Cary, NC). Plasma RNA data were subjected to logarithm transformation before statistical analysis. Spearman correlation coefficients were calculated by the SAS statistical analysis software package. Plasma RNA data were subjected to logarithm transformation before statistical analysis.

Figure 8 illustrates QC-PCR results from a control reconstruction experiment, along with a representative experimental determination of plasma HIV-1 copy number for an infected patient. Virion-associated RNA was detected and quantified in plasma specimens from all 66 HIV-1-infected subjects (Table 5). Determined RNA copy numbers ranged from 1.00×10^2 to 2.18×10^7 HIV-1 RNA copies per milliliter of plasma (corresponding to 0.50×10^2 to 1.09×10^7 virions per milliliter). Positive signals for wild-type HIV-1 target sequences were not observed in specimens from any of ten uninfected control subjects or, when the reverse transcription step was omitted, for specimens from any of the HIV-1-infected patients.

The threshold sensitivity for RNA QC-PCR analysis was determined to be 100 copies per reaction. Although positive signals could be detected for as few as 10 to 20 copies, results were less consistent below 100 copies per reaction, and quantitation was less reliable. QC-PCR analysis was typically performed with 0.5 ml plasma samples. However, pelleting the virus from increased volumes of plasma for specimens containing low numbers of virions did allow for increased overall sensitivity of detection. Thus, for one p24 antigen-negative, culture-negative CDC stage II patient

(Patient TIM 0852, Table 1), initial QC-PCR analysis of 0.5-ml specimen portion gave a negative result. However, analysis of virus pelleted from 2.8 ml of a replicate plasma sample allowed unequivocal detection of the low amount of virus present in this patient's plasma (100 copies per milliliter), whereas no HIV-1 RNA was seen with analysis of comparable volumes of plasma from HIV-1 seronegative controls. In separate control studies, analysis of triplicate portions of plasma samples from six different patients, with mean HIV RNA copy numbers of 6.7×10^4 to 1.0×10^6 per milliliter, gave a mean SD of 22%. Analysis of replicate portions of the same HIV-1 RNA preparation on six different days gave an SD of 15%. This reproducibility contrasts with variability of up to 600 to 1000% reported for noncompetitive PCR procedures.

Whereas the QC-PCR method quantified virion-associated HIV-1 RNA in all 66 patients tested, virus culture and standard p24 antigen assays were much less sensitive, with positive results in 4/20 and 5/20 subjects with $CD4^+$ T cell counts >500 per cubic millimeter, 6/18 and 7/18 subjects with $CD4^+$ T cell counts of 200 to 500 per cubic millimeter, and in 22/28 and 24/28 subjects with $CD4^+$ cells fewer than 200 per cubic millimeter, respectively (Table 5). In 30 patients with negative results in standard p24 antigen assays, the use of acid treatment to dissociate p24 from immune complexes increased the frequency of detection by only four patients, although absolute measured amounts of p24 were increased in 25/36 patients with detectable levels of p24 before acid treatment.

QC-PCR-determined HIV-1 RNA levels in plasma differed between clinical stages, with levels for CDC stage II and III patients (asymptomatic or persistent lymphadenopathy) [mean, 78,200 copies per milliliter (n=22)] significantly lower than for CDC stage IVC2 ([AIDS-related complex (ARC)] [mean, 352,100 copiers per milliliter (n=23); $P \leq 0.05$], and RNA levels for CDC stage IVC1 (AIDS) patients had circulating virus levels comparable to those observed during the

peak of viral replication in stage I (primary infection) patients [mean, 5,178,000 copies per milliliter (n=6); difference not significant], implying that late-stage disease is characterized by a nearly complete loss of immunological control of viral replication. There was also a significant correlation between increasing QC-PCR-determined HIV-1 RNA levels and decreasing absolute $CD4^+$ T cell counts [Spearman rank correlation coefficient $r=-0.765$, $P < 0.0001$]. A nonlinear regression analysis of these data yielded the equation log (RNA) = 4.43 + 1.77exp[-0.0049(number of $CD4^+$ T cells)], with an r^2 value of 0.56 ($P < 0.0001$).

Figure 9 shows data for longitudinal specimens collected from three individuals [of six studied (Table 5)], beginning at the time of presentation with acute HIV-1 infection (CDC Stage I) and continuing through the establishment of chronic infection (CDC stage II to IVC2). Each patient presented with signs and symptoms of primary infection and with no detectable HIV-1-reactive antibodies as determined by enzyme-linked immunosorbent screening assay or protein immunoblot, and each subsequently seroconverted with a fully spectrum of HIV-1-specific antibodies by day 50. Virion-associated HIV-1 RNA levels peaked between 8 and 23 days after the onset of symptoms, reaching valued between 3.55×10^5 and 2.18×10^7 copies per milliliter (corresponding to 1.78×10^5 to 1.09×10^7 virions per milliliter). Culturable virus [10 to 10,000 tissue culture infectious dose (TCID) per milliliter] and p24 antigen (258 to 5,406 pg/ml) peaked at approximately the same time as virion RNA levels, declining rapidly thereafter in parallel with virion RNA levels. Within the first 100 days after onset of symptoms plasma RNA levels fell by between 20- and 235-fold from peak levels but, in marked contrast to p24 antigen and culturable virus, remained continuously quantifiable for the duration of follow-up in all patients.

Among three patients who presented with symptomatic acute infection (Fig. 9), there appeared to be associations between profiles of viral load in plasma over time, as determined by

RNA QC-PCR, and trends in CD4$^+$ T cell counts and clinical status. For example, patient SUMA (Fig. 9A) showed an initial peak of HIV-1 RNA (1.49 x 10^6 copies per milliliter) that decreased by a factor of nearly 1 x 10^2 within the first month of follow-up. Viral RNA reached a minimum (2.30 x 10^3 copies per milliliter) at 278 days and remained at or below 1.50 x 10^4 RNA copies per milliliter up to 473 days of follow-up. Plasma viral cultures and p24 assays remained negative after the initial peak. This patient maintained a normal CD4$^+$ T cell count (952 to 1108 per cubic millimeter) and remained entirely asymptomatic over the period of follow-up. In contrast, patient FASH (Figure 9B) showed the highest initial virus peak (2.18 x 10^7 copies per milliliter), as well as the highest persistent levels of circulating virus, in the range of 3.26 x 10^5 to 6.32 x 10^5 copies per milliliter for 301 days of follow-up. The highest CD4$^+$ T cell count measured in this patient was 282 per cubic millimeter, with a subsequent progressive decline to 128 per cubic millimeter and clinical progression to CDC stage IVC2, despite antiviral therapy. Although anecdotal, these observations suggest that higher levels of circulating virus and failure to effectively control virus replication after initial infection may be associated with a negative prognosis. However, in addition to quantitative viral load, other virologic or immunopathologic factors, including a syncytium-inducing viral phenotype, likely contribute to variable rates of CD4$^+$ T cell depletion and clinical outcome (24). In this regard, patient WEAU (Fig. 9C), who experienced a sharp decline in CD4$^+$ cells despite a peak level of viral RNA of only 3.55 x 10^5 copies per milliliter, was infected with a virus isolate that was markedly cytopathic when cultured in vitro (24).

Although in the present studies we did not intend to provide a comprehensive evaluation of the impact of therapeutic intervention on viral load as measured by QC-PCR, we did analyze sequential specimens from a limited number of patients, before and after initiation of treatment with azidothymidine (AZT). As shown in Table 6, treatment with AZT resulted in up to 39-fold

decreases in circulating virus as measured by QC-PCR (Patient JOJI 0070), with a significant treatment effect for the entire group of patients ($P < 0.0001$). Despite differences between the absolute levels of virus measured by QC-PCR and by culture methods, treatment-associated decreases in plasma HIV-1 RNA levels were paralleled by decreases in culturable virus, where measured. We also evaluated in greater detail serial specimens from three previously untreated patients who were given AZT for 6 weeks, followed by a 1-week period off treatment. The observed rapid decreases in QC-PCR-determined circulating virus levels after initiation of treatment (week 1) and the rapid rebound of virus to pretreatment levels after discontinuation of treatment (week 7) reveal the dynamic nature and high levels of ongoing viral replication in these patients. Two of these patients completely lacked detectable levels of other viral markers and could only be monitored by QC-PCR, and the third had detectable levels of p24 only after ICD (Table 6). The average decline in HIV-1 RNA among the ten patients treated with AZT was 11-fold, whereas the average decline associated with resolution of the acute retroviral syndrome in six patients was 72-fold (Fig. 9 and Table 6).

Using different PCR techniques, other investigators have estimated levels of HIV-1 RNA in plasma ranging from 0 to 1×10^6 copies per milliliter (reviewed in 32), values that are generally lower by a factor of 10 or more than those we observed for patients with disease of a comparable stage. The methods used by these investigators differed in various significant ways from those we describe here, including, in some instances, the use of noncompetitive PCR techniques that do not provide the level of stringent internal control that is a central feature of QC-PCR, the use of different target regions and primer sets, or direct extraction of RNA from plasma without pelleting of virions by ultracentrifugation and other steps to maximize recovery of viral RNA.

Validation of the QC-PCR procedure with the use of known copy numbers of recombinant

HIV RNA and DNA (Fig. 1) and by measurement of viral RNA in virus preparations that had been quantified directly by electron microscopic particle counts makes it unlikely that we have overestimated total virion level in patient samples. To demonstrate conclusively that the HIV-1 RNA quantified by QC-PCR was virion associated, we fractionated samples of HIV-1-containing culture supernatant and plasma from infected patients by using buoyant density centrifugation on continuous (20 to 60%) sucrose gradients. The HIV-1 RNA peaks corresponded precisely to the peaks of HIV-1 p24 antigen, both of which localized to fractions of the expected specific gravity for HIV-1 particles. For the banded virus, the ratio of virions (assuming two HIV-1 RNA molecules per virion) to p24 antigen (in picograms) was approximately 10^4:1, in good agreement with estimates based on other biophysical studies of HIV-1 virions (33). Similar ratios were seen for virus pelleted from plasma (Table 5), with QC-PCR-determined virus RNA levels for most subjects exceeding p24 levels (measured in picograms) by the expected 1,000- to 10,000-fold.

Circulating levels of plasma virus determined by QC-PCR also correlated with, but exceeded by an average of nearly 60,000-fold (Table 5), titers of infectious HIV-1 determined by quantitative endpoint dilution culture of identical portions of plasma. Several virologic and immunologic factors already identified in HIV-1 infection, including neutralizing antibody, viral envelope shedding, deterioration of other viral components, and genotypically defective virus (reviewed in 34) likely contribute to the differences in levels of circulating virus determined by QC-PCR and titers of culturable virus. However, the minimum requirements for establishment of productive infection of primary mononuclear cells are not known. If more than one intact viral particle is required to attain productive infection of a host cell, this would exaggerate the discrepancy observed between viral titers in plasma determined by QC-PCR as compared with those determined by endpoint dilution culture. For HIV-1 propagated in vitro, total virions have

been reported to exceed culturable infectious units by factors of 10^4 to 10^7 (33), ratios similar to those we observed in plasma.

The evidence provided here for continuous viral replication throughout all stages of infection and the demonstration of significant associations between HIV-1 RNA levels and both disease stage and $CD4^+$ T cell counts argue strongly for a direct role for HIV-1 replication in the pathogenesis of HIV disease. These results confirm and extend work by Coombs (19), Ho (20), Pantaleo (30), Schnittman (10, and Michael (35) who, by alternative approaches, also found evidence of continuous viral replication related to clinical stage and disease progression. The finding of a large proportion of circulating virus that is not culturable raises the possibility that genetically defective (36) or otherwise noninfectious virus may contribute importantly to HIV-1 pathogenesis, in keeping with precedents in animal retrovirus systems. Numerous mechanisms by which noninfectious particles might contribute to the pathogenesis of HIV-1 infection have been proposed (reviewed in 32). Furthermore, recent observations have documented the presence of HIV-1-reactive cellular immune responses in patients who have been exposed to HIV-1 by sex or use of shared needles but who are not demonstrably infected with the virus (37). Exposure to an inoculum consisting largely of noninfectious particles might produce such a result, even without the establishment of productive infection of the exposed individual.

Finally, our data suggest that determination of virion-associated HIV-1 RNA levels in plasma by QC-PCR represents a marker of viral replication with potential for wide-spread applicability in assessment of the activity of antiretroviral therapy. There is currently a widely recognized need for new markers that allow timely assessment of the in vivo antiviral activity of new therapeutic approaches and agents and that better predict the ultimate clinical efficacy of new treatments. Ideally, such markers should bear a biologically plausible relation to the disease

process, be present in most or all patients change rapidly in response to effective therapy, be derived from readily obtained clinical specimens, and correlate directly with eventual clinical outcome. Quantitation of HIV-1 virion levels in plasma as determined by QC-PCR satisfies the first four of these requirements. Future studies making use of PC-PCR methods will help to determine the relation between virion-associated HIV-1 RNA levels and clinical outcome and the role of persistent viral replication and viremia in the pathogenesis of HIV infection and AIDS.

4. Quantification of HIV-1 In Vivo by Culture, Competitive PCR, and p24 Antigen Determinations Before and After Therapy With Nucleoside and Non-nucleoside Reverse Transcriptase Inhibitors Therapy.

Current treatment of human immunodeficiency virus type 1 (HIV-1) infection consists exclusively of the nucleoside analog reverse transcriptase inhibitors, zidovudine, didanosine, and zalcitabine. Unfortunately, the antiretroviral and clinical effectiveness of nucleoside therapy diminishes over time. It remains unclear whether the loss of effectiveness of these agents is due to incomplete inhibition of viral replication, development of resistant viral variants, or other mechanisms of disease pathogenesis. Therefore, the search for new agents which more completely inhibit viral replication, preferably via complementary mechanisms of action, has intensified.

A new group of nonnucleoside reverse transcriptase inhibitors has been developed over the last several years (38-41). In contrast to the nucleoside analogues, which inhibit the reverse transcription process via incorporation into the elongating DNA strand with resultant chain termination, the nonnucleoside agents act through direct inhibition of reverse transcriptase and are not incorporated into the growing DNA chain. These agents, which include the tetrahydroimidazobenzo-diazepinone (TIBO) derivatives, alpha-anilino phenylacetamide derivatives, ateviridine (U-90152), nevirapine (BI-RG 587), and the pyridinone derivatives (38-41), selectively

inhibit HIV-1 (but not HIV type 2) reverse transcriptase at nanomolar concentrations and are active against HIV-1 isolates that are resistant to zidovudine.

Recently, one compound from the pyridinone group, L-697,661 (41), was selected for further development in Phase I/II clinical trials. Preclinical studies and early pharmacokinetic studies in man demonstrated good oral bioavailability, with serum levels (Cmax) of greater than 1 μM after a single 500 mg oral dose, and an acceptable safety profile. The availability of this novel potent HIV-1 RT inhibitor along with zidovudine provided an important opportunity to evaluate QC-PCR and other quantitative virologic measures as indicators of clinically meaningful antiviral drug activity.

Two independent, concurrently run clinical trials were initiated in May, 1991 at The University of Alabama at Birmingham (UAB). Each protocol was approved by the UAB Institutional Review Board. Protocol A evaluated HIV-1 infected patients with CD4 counts between 200 and 500 cells/mm3 and Protocol B studied patients with CD4 counts < 200 cells/mm3. Entry criteria for both protocols included: age > 17 years; men and non-pregnant women; normal renal and hepatic laboratory values (all < twice the upper limit of normal); seronegativity for hepatitis B surface antigen; no active recreational drug or alcohol abuse; and ability to give informed consent. Patients in Protocol A were excluded if they had a previous AIDS defining condition. Patients in Protocol B were allowed into the study if they had previous Pneumocystis carinii pneumonia or stable Kaposi's sarcoma but were ineligible if they had a history of any other AIDS-defining condition. Prior antiretroviral therapy with zidovudine was allowed, however, all patients had to be off therapy for a minimum of 14 days prior to receiving study medication. No investigational or immunosuppressive agents were permitted within 30 days of study entry.

The studies were double-blind, randomized, parallel-group six week clinical trials. In both

protocols, patients were assigned according to a randomized allocation schedule into one of four treatment groups: Group 1, low-dose L-697,661 (25 mg orally every 12 hours); Group 2, middle-dose L-697,661 (100 mg orally every 8 hours); Group 3, high-dose L-697,661 (500 mg orally every 12 hours); or Group 4, standard therapy with zidovudine (100 mg orally every 4 hours, 5 times daily). Fifteen evaluable patients were sought for each of the four treatment groups in both protocols (total target enrollment 120 patients). At the end of the six week study period, study drugs were stopped for a one week "washout" period during which additional surrogate marker tests of antiviral activity were obtained. After this period, the patients were allowed to continue taking study medications as part of an extension protocol in a double-blind fashion. Protocol B participants continued therapy for an additional 6 weeks according to their originally assigned regimens. Patients in Protocol A on L-697,661 continued to receive drug according to their original treatment assignments; however, Protocol A patients originally assigned to zidovudine were randomly reassigned to receive one of the three doses of L-697,661. Within the two weeks prior to study enrollment, volunteers were evaluated with a history and physical examination, an electrocardiogram, and clinical laboratory evaluation. Baseline laboratory studies and physical examination were repeated on the day of study entry and on each subsequent study visit as part of the safety analysis. Patients were assessed on a weekly basis throughout the six week study period and again at week seven after the one week washout period. During the six week extension, patients were evaluated every other week.

Antiretroviral activity was assessed via weekly CD4 cell counts and HIV-1 p24 antigen (Abbott Laboratories, Chicago, ILL.) determinations. A positive HIV p24 antigen test was defined as one that detected > 4 pg p24 antigen per milliliter. As part of the protocol, aliquots of plasma and peripheral blood mononuclear cells (PBMCs) were collected periodically throughout the study

and stored at -70°C ml and -156°C, respectively, for future use, including evaluation of resistance development. Blood for virus isolation was obtained from all patients on the day therapy was initiated (day 0) and again at day 49, one week after therapy was discontinued. Pre- and post-therapy virus isolates from 22 randomly chosen patients were selected for evaluation. HIV-1 was isolated from patient PBMCs by co-cultivation with PHA-stimulated donor PBMCs as described previously (18). Thus, all virus isolations represented primary virus amplifications.

Paired primary virus isolates (pre- and post-treatment) were thawed and added separately to 5.0×10^6 activated, uninfected normal donor PBMCs in 5.0 ml of culture medium. Additional activated PBMCs were added at two day intervals resulting in a final culture, at seven days after initiation, that contained approximately 4.0×10^6 cells/ml in 40 ml. Following an additional two days of incubation, the culture medium was collected, centrifuged to remove residual cells, and stored at -70°C in 1.0 ml aliquots. Viral p24 antigen content of the stored medium was determined using a commercial assay (Coulter, Hialeah, FL).

Sensitivity assays were performed using 48-well cell culture plates. Each well contained 5.0×10^5 activated, uninfected human PBMCs in a total volume of 0.5 ml culture medium. Identical virus inocula (5-500 pg p24 antigen) were used for both pre- and post-treatment isolates. Test compounds were added to the culture wells in a two-fold dilution series. Twenty-four hours after the start of the assay, the virus inoculum was removed by harvesting and washing the cells from each well. The cells were then resuspended in fresh medium containing the appropriate concentration of test compound and were seeded in 96-well cell-culture plates. Each well received 2.5×10^5 cells in 0.25 ml. Cultures were fed with compound-containing medium and viral p24 antigen levels were determined every 2-3 days.

All assays were performed in quadruplicate and control cultures without test compound

were included. The 90% inhibitory concentration (IC90) was determined on day 11 of the assay by comparing the mean p24 antigen levels in test cultures with the levels expressed by the control cultures. The IC90 value was the lowest actual concentration of test compound that inhibited p24 antigen expression by at least 90 percent.

DNA was extracted from the cryopreserved PBMCs (approx. 1.0-5.0x10^6 cells) from the virus isolate expansion. The viral reverse transcriptase gene (1680 bp) was amplified using nested primer PCR. The flanking primer pair was: (sense) 5'GGACCTACACCTGTCAACAT (nucleotides 400-419 of HXB2) and (antisense) 5'TCACTAGCCATTGCTCTCCA (nucleotides 2194-2218). The nested primer pair was (sense) 5'CCGACCTGCATAGAATTCATGCC(C/A)ATTAGTCCTATTGA (nucleotides 466-482) and (antisense) 5'GCCCCGACCTGCAT AAAGCTTATAGTAC(C/T)TTCCTGATTCC (nucleotides 2127-2144). The nested primers included either ATG initiation or TGA termination codons and restriction enzyme sites to facilitate cloning and expression of reverse transcriptase. The first round PCR reaction mix contained 1X PCR buffer I (Perkin Elmer Cetus, Norwalk, CT), 200 μM each dNTP, 1 μM each flanking primer, 0.5 μg template DNA and 2 units Taq polymerase in a 50 μl final volume. The amplification conditions were: 1 min at 94°C, 1 min at 56°C, and 3 min at 72°C for 25 cycles. Upon completion, 3 μl of this reaction were added to a second round PCR mix, which contained 1 μM of each nested primer, in a 100 μl reaction. PCR cycling parameters were: 1 min at 94°C, 1 min at 45°C, and 3 min at 72°C for 5 cycles. Then the annealing temperature was raised from 45°C to 52°C, and the samples were amplified for an additional 30 cycles. The reverse transcriptase PCR products were cloned into the EcoRI and HindIII sites of bacterial expression plasmid pLG18-1, a derivative of pRT1-lacI (42) in which the reverse transcriptase coding sequences were replaced with polylinker HindIII/EcoRI Genblock (Pharmacia LKB Biotechnology,

Piscataway, NJ). The expression plasmids were screened for the presence of a functional reverse transcriptase using the in situ assay, essentially as described by Prasad and Goff (43). This was done to eliminate the sequencing of clones containing stop codons. Reverse transcriptase expression was induced with 200 μM isopropyl-ß-D-thiogalactopyranoside for 6 hours. The reverse transcriptase coding region from three molecular clones which exhibited enzymatic activity in the in situ assay was sequenced. Sequence analysis was confined to the N-terminal 343 codons of the reverse transcriptase gene.

QC-PCR analyses and infectious virus titers in plasma were performed as described above. Differences in baseline characteristics among treatment groups were assessed with an extension of Fisher's Exact test for categorical outcomes and analysis of variance (ANOVA) for continuous outcomes (on ranks for CD4 and p24). Safety and tolerability were assessed by tabulating clinically relevant adverse experiences. Fisher's Exact test was used to compare the observed incidence rates among and between the treatment groups. Adverse experiences were recorded only if their onset occurred between the time of first dose and the end of the extension period.

Mixed-effect models (44,45) were used to analyze the temporal change in each surrogate marker over the first six weeks of treatment. The analysis focused on the first six weeks due to the change to L-697,661 therapy among zidovudine recipients in Protocol A. The logarithm of the ratio to baseline between week 1 and week 6 was modeled as a simple, random slope over time within a patient. The p24 analysis was restricted to patients with a p24 antigen value above the detectable limit at pretreatment (i.e. > 4 pg/ml). Nondetectable values in subsequent visits were set to the minimal detectable level. The two protocols were analyzed separately except for p24 antigen where the few patients with detectable levels in Protocol A (N=18) were analyzed with the Protocol B patients. Statistically significant results ($p \leq 0.05$) were reported without adjustment

for multiple comparisons and all P-values were based on two-sided tests.

A total of 68 patients in Protocol A and 67 patients in Protocol B were randomly assigned to one of the four treatment regimens. Only two patients in each study were noncaucasians. The patients were predominantly male (93 percent) and the average age was 36 years. No significant demographic differences between treatment groups were detected in either study. Furthermore, no clinically important differences were seen between treatment groups with respect to pretreatment laboratory safety parameters. As expected, a greater proportion of patients in Protocol B (69 percent) had detectable p24 levels recorded at the pretreatment timepoint than in Protocol A (26 percent). In Protocol A, significant differences in median pretreatment CD4 counts were evident between patients treated with 50 mg L-697,661 (395/mm3) compared with those receiving 300 mg L-697,661 (243/mm3; P = 0.009), 1000 mg L-697,661 (294/mm3; P = 0.050), and zidovudine (294/mm3; P = 0.024). No within-protocol treatment differences were detected in pretreatment p24 antigenemia.

Among the participants in Protocols A and B, 59 (87 percent) and 55 (82 percent), respectively, had received previous zidovudine therapy. The majority of zidovudine experienced patients (38/59 and 45/55 in Protocols A and B, respectively) had received greater than six months of therapy. In both studies, a greater proportion of patients assigned to the low dose L-697,661 treatment regimen were zidovudine experienced, but this difference was not significant.

Eight patients (12 percent) in Protocol A and 18 patients (27 percent) in Protocol B did not complete the full 13 week study period. An additional patient in Protocol B discontinued treatment shortly before the week 6 visit because of elevated liver function tests, but was rechallenged after the washout period without subsequent hepatic dysfunction. Only two patients in Protocol A were discontinued for clinical adverse experiences. These included nausea (2), headache (1), and

lethargy (1). Ten patients in Protocol B were discontinued for adverse clinical experiences. The reasons for discontinuation included: esophageal ulcer/candidiasis (1), pulmonary cryptococcosis (1), worsening Kaposi's sarcoma requiring chemotherapy (1), desquamating skin rash (2), constitutional symptoms (3), cerebral toxoplasmosis (1), and peripheral neuropathy (1). Three clinical adverse events in combination with elevated transaminase tests were noted, including one patient with Hodgkin's lymphoma (Protocol A), one patient with rash, and another patient with fever (Protocol B). Two patients (one in each protocol) were discontinued within the first week of the study when it was discovered they did not meet entry criteria and were ineligible.

There were no differences in the frequency of fever, headache or asthenia/fatigue. However, significantly more patients in the 300 mg L-697,661 arm experienced skin rashes than in the zidovudine arm (P = 0.045). More patients reported at least one episode of nausea in the zidovudine arm than in the other arms, but this difference was not significant. One patient receiving L-697,661 (1,000 mg) in Protocol A had treatment temporarily discontinued due to elevated liver enzymes (AST and ALT), but was rechallenged with a lower dose (300 mg/d) of drug with no subsequent recurrence of hepatic dysfunction. No patient had significant elevation in total bilirubin or serum creatinine levels. A hemoglobin value of 8 mg/dl or less was not observed in any Protocol A patient, but was seen in four Protocol B patients - one per treatment group. No patient had a white blood count below 1000/mm3. Two patients developed a serious opportunistic infection or malignancy before the conclusion of 13 weeks of study: one had Hodgkin's lymphoma (50 mg L-697,661, Protocol A) and the other had cerebral toxoplasmosis (50 mg L-697,661, Protocol B). One patient died within the 13 week study period, but had been discontinued from the study for over two weeks (zidovudine, Protocol B).

Mixed model estimates for percent change from baseline in CD4 count after one week and

six weeks of treatment are shown in Table 7. None of the CD4 count changes from baseline were significant among Protocol A patients. For Protocol B patients, significant increases from baseline were seen at week 1 in the 300 mg L-697,661 group (+53.2 percent; $P < 0.001$) and in the 1000 mg L-697,661 group (+29 percent; $P = 0.012$), although the responses in each group were lost by week 6 (+6.7 percent and -2.7 percent, respectively). In contrast, patients receiving low dose L-697,661 experienced an immediate and progressive fall in CD4 counts compared to baseline, reaching statistical significance by week 6 (-32.1 percent; $P = 0.002$). CD4 counts did not change significantly from baseline among zidovudine recipients (4.8 percent wk 1, 4.6 percent wk 6; $P > 0.05$ for both).

Mixed model estimates for percent change from baseline in HIV-1 p24 antigen at week 1 and week 6 are also shown in Table 7. The 50 mg, 300 mg, and 1000 mg L-697,661 groups all experienced significant declines in p24 antigen below baseline at week 1 (-19.2 percent, $P = 0.042$; -31.9 percent, $P = 0.002$; -42.1 percent, $P < 0.001$; respectively). However, only the 300 mg L-697,661 group differed from baseline at week 6 (-34.1 percent, $P = 0.015$). A trend toward decreased p24 antigen values was observed in the zidovudine group, but this did not reach statistical significance. In the 1000 mg L-697,661 group, the slope estimate of p24 antigen between week 1 (-42.1 percent) and week 6 (-17.0 percent) was statistically significant ($P < 0.05$), thus raising the possibility of viral drug resistance development.

In Table 8, the IC90 values for L-697,661 and zidovudine are presented for viral isolates from 19 randomly selected patients before and after treatment. All pre-treatment (week 0) virus isolates were found to be sensitive to L-697,661 with IC90's that ranged from 25 to 800 nM. Loss of sensitivity to L-697,661, defined as an 8-fold or greater difference between pre- and post-treatment isolates, was observed in 5/5 patients receiving high-dose therapy (1000 mg/day);

in 4/5 patients receiving medium-dose therapy (300 mg/day); and in 2/6 patients receiving low-dose therapy (50 mg/day). The development of drug resistance generally coincided with increases in plasma p24 antigen between weeks 1 and 6 of treatment (Figure 10). As expected, changes in sensitivity to zidovudine were not found in virus from the L-697,661-treated patients, nor were changes in L-697,661 sensitivity observed in virus isolates from patients treated with zidovudine.

The reverse transcriptase coding regions of the 19 isolate pairs were sequenced and analyzed for amino acid substitutions known from in vitro studies to contribute to the L-697,661 and zidovudine resistant phenotypes (41). Three molecular clones from each isolate representing the predominant viral genotype were sequenced. The results, summarized in Table 8, reveal a perfect correlation between reduced viral sensitivity to the nonnucleoside inhibitor and the selection for mutational alterations at reverse transcriptase residues 103 and/or 181. Eight- to ten-fold loss of L-697,661 sensitivity was associated with a substitution of glutamine for lysine at position 103 (patient #428). Higher level resistance (≥ 30-fold) was associated exclusively with a cysteine for tyrosine substitution at 181 that was occasionally co-expressed with an asparagine for lysine change at 103. An arginine for lysine substitution at position 103 noted in virus from patient #466 was not associated with reduced drug sensitivity. Resistance to zidovudine correlated with alterations at residues 41, 67, 70, 215, and 219 as (reviewed in 46).

The pre- and post-therapy virus isolates from patients 115,139, and 428 were tested for cross-resistance to other nonnucleoside reverse transcriptase inhibitors (L-697,229; BI-RG-587; R82913). Compared to pre-therapy isolates which were uniformly sensitive to these agents, the post-therapy viruses were resistant to all three inhibitors, showing increases in IC90's between 15 and 240-fold (data not shown).

This study is the first report of the clinical activity and safety profile of L-697,661, a novel

antiretroviral agent representative of the nonnucleoside reverse transcriptase inhibitors. All three doses of L-697,661 were well tolerated and comparable to zidovudine in safety profile. Over the 13 week study period, a small proportion of patients discontinued therapy due to an adverse experience. With few exceptions, these were evenly distributed across all study groups and between the two protocols. Nausea was a common side effect and occurred more often among zidovudine recipients. Skin rash was the only adverse experience that occurred more frequently among L-697,661 recipients. Although preclinical studies indicated potential hepatotoxicity of L-697,661, very few patients in the L-697,661 groups had significant elevations of transaminase levels. The frequency of transaminase abnormalities did not differ significantly between L-697,661 and zidovudine treated subjects.

Previous studies with zidovudine and other nucleoside agents generally have observed a significant rise in CD4 count over the first 6 to 8 weeks of therapy with a return toward baseline by 24 weeks. In this study, CD4 counts increased significantly at week 1 in the medium and high-dose L-697,661 groups, but returned to baseline by week 6 (Table 7). The low-dose L-697,661 treated group experienced an immediate and progressive decline in CD4 numbers. The zidovudine treated patients experienced no significant change in CD4 counts over the treatment period, probably due to the fact that over 80 percent of the patients enrolled in this trial had received prior zidovudine therapy.

Changes in plasma p24 antigen levels generally correlated inversely with changes in CD4 counts with a significant early (week 1) but unsustained fall in response to L-697,661 therapy (Table 7). In many patients, the fall in p24 antigen at week 1 was followed by a progressive rise in p24 antigen to baseline or even higher values (Figure 10), suggesting that viral drug resistance had developed.

Previous studies of HIV-1 isolates exposed to L-697,661 in vitro demonstrated a marked reduction in viral susceptibility to the drug in association with single point mutations at either of two critical amino acid positions (103 and 181) within the reverse transcriptase gene product. Based on these findings, pre- and post-therapy isolates from a subset of patients were analyzed for susceptibility to L-697,661 and their reverse transcriptase genes were sequenced. Results of this analysis showed that treatment with L-697,661 indeed resulted in the selection of HIV-1 variants that were resistant to inhibition by the compound. Virus sensitivity to L-697,661 was not influenced by prior treatment with zidovudine and, conversely, changes in virus sensitivity to zidovudine were not observed in patients following treatment with the nonnucleoside inhibitor. Nucleic acid sequence analysis of the post-therapy isolates revealed alterations at positions 103 and 181 in virus strains that exhibited drug resistance but not in those which remained drug sensitive. The frequency of resistance development was greater in patients treated with medium (4 of 5) and high (5 of 5) doses of L-697,661 than in those treated with lower doses (2 of 6). The lack of development of resistance at the lowest dose of L-697,661 is most likely due to insufficient selective pressure. Taken together with the CD4 and p24 antigen responses (Table 7), the data argue for a clinically important dose-related antiviral effect of L-697,661.

The observed rapidity with which resistant virus populations were selected reflects a previously unsuspected dynamism of HIV-1 in vivo. Although there has been a growing appreciation that microbiological latency of HIV-1 in vivo does not exist (30,32), our study suggests an even higher rate of ongoing viral replication, and presumably de novo cellular infection, than previously appreciated. Recent studies using PCR to detect and quantify virion-associated RNA in plasma corroborate this finding by demonstrating continuous HIV-1 replication throughout all stages of infection (32). Coffin has shown in studies of avian leukosis

virus and by computer modeling that even a small (4.0 percent) growth advantage for a variant virus can result in complete replacement of the parental population within 40-50 replication cycles (47). These findings likely explain the rapid emergence of viral resistance to L-697,661 in our patient population. A heightened appreciation of viral dynamics will be important in the design and interpretation of future clinical evaluations of HIV-1 specific antiviral compounds.

The rapid selection of viral variants with decreased sensitivity to L-697,661 will limit the clinical usefulness of this and probably other nonnucleoside reverse transcriptase inhibitors. However, in vitro studies have shown additive or synergistic activity between nucleoside and nonnucleoside antiretroviral agents. Thus, these agents may still find utility in combination regimens. Finally, since the emergence of resistant isolates occurred in this study in the setting of established infection, when viral genetic complexity is extensive and subpopulations of resistant virus are more likely to exist, the use of nonnucleoside agents in very early infection or as post-exposure prophylaxis may be especially advantageous.

5. <u>Molecular and Biologic Characterization of HIV-1 *in vivo*.</u>

The premise of this work was that transmission and replication of HIV-1 (and HIV-2) in natural settings may result from complex interactions between replication competent and defective viruses and that the cell tropisms and replication patterns of these viruses *in vivo* may not be reflected fully by tissue culture. Using techniques for DNA extraction, recombinant lambda phage cloning, PCR amplification, DNA sequencing, and DNA transfection (48), we sought to obtain full-length proviral clones from *uncultured* human brain.

Patients with AIDS dementia complex (ADC) are known to harbor considerable amounts of HIV-1 sequences within their brain, both in chromosomally integrated as well as unintegrated form (94,50). While both linear and circular viral molecules contain a complete viral genome, viral

circles can be cloned in their entirety after linearization with an appropriate restriction enzyme. The abundance of circular viral DNA in the CNS of certain patients with AIDS dementia complex therefore prompted us to attempt molecular cloning of replication-competent HIV-1 genomes directly from this viral population.

Screening several brain DNAs from AIDS dementia patients, we identified one sample to be particularly suitable for lambda phage cloning. This DNA preparation was available in sufficient quantities, exhibited no signs of degradation, and contained enough HIV-1 viral sequences to be visualized by Southern blot analysis. Restriction enzyme mapping with EcoRI and SstI (alone and in combination) confirmed that EcoRI cleaved the provirus only once and indicated that viral circles comprised the majority of HIV-1 sequences within this particular sample (Figure 11). We therefore constructed a genomic lambda phage library in λ gt wes.λB using EcoRI digested brain DNA fractions 9 to 12 Kb in length (Figure 11). Screening approximately 30 genomic equivalents (8×10^6 lambda phage plaques), we identified ten recombinant clones which hybridized to a probe specific for HIV-1.

Detailed restriction enzyme analysis and Southern blot hybridization studies confirmed the presence of HIV-1 sequences in all ten recombinant clones and determined the length and orientation of their viral inserts (Figure 12). Nine of ten clones represented unintegrated linearized circles which were cloned in permuted form at a single EcoRI site located in the central viral region. The restriction enzyme cleavage pattern of a tenth clone (YU-6) suggested the presence of an integrated provirus (5' half) flanked by normal cellular sequences. Side-by-side comparison with a prototype HIV-1 construct known to possess a complete proviral genome (pHXB2D) identified four brain clones to comprise full-length HIV-1 inserts with either one (YU-2, YU-10, YU-21) or two (YU-32) tandem copies of the viral LTR. Three clones contained internal deletions

(YU-1, YU-5, YU-27), while two other clones (YU-3, YU-4) exhibited genomic rearrangements which were consistent with the presence of an extraneous LTR in reverse orientation.

Nucleotide sequence analysis was performed to further characterize all clones in regions which could not be definitively mapped by restriction enzyme analysis alone. Internal deletions of varying length were identified in three molecular clones (Figure 12 and 13). YU-5 contained a 3044bp deletion in the 3' half of its genome, which included portions of *tat* and *rev*, the entire *vpu* and *env* gene, as well as the N-terminal half of *nef*. YU-27 was characterized by a less extensive deletion (549 bp) which included the C-terminus of the env gene and the N-terminus of the *nef* gene. Yu-1 contained a 555 bp deletion in its *gag* gene which involved the entire p17 and a portion of the p24 protein, but did not include sequences 5' of gag such as the viral packaging signal. None of the deleted fragments extended to the U3 or U5 region of the viral LTR. The EcoRI cloning site was mapped within the *vpr* gene (position 5742 of the HXB2D sequence).

Integration of most retroviruses involves the removal of 2 base pairs from the termini of the unintegrated linear DNA precursor, as well as a duplication of the target sequence at the insertion site (51-53). Viral circles with two LTRs generally contain those nucleotides which are lost during the integration process within their circle junction since they result from blunt-end ligation of unintegrated linear molecules. To examine whether HIV-1 proviral integration in primary tissue involved similar mechanisms, we sequenced the LTR-LTR circle junction in clone YU-32 (Figure 13). The results demonstrated the presence of four additional nucleotides between the conserved CA and TG dinucleotide that generally delineate the boundaries of integrated HIV-1 proviruses (..CA-GTAC-TG..). These data thus indicated that linear HIV-1 molecules contain two dinucleotides at each terminus which are lost before or during recombination with the host genome *in vivo*.

Nucleotide sequence analysis was also required to further characterize the nature of viral and non-viral sequences in clone YU-6. Restriction enzyme analysis indicated several enzyme site differences 5' of the YU-6 LTR which distinguished this phage clone from all other brain derived constructs. Subgenomic probes of HIV-1 failed to detect envelope sequences 5' of the LTR, whereas this same region hybridized to nick-translated normal human PBMC DNA thus indicating the presence of human repetitive sequences (data not shown). Nucleotide sequence analysis of the LTR junction fragment of YU-6 identified the expected boundaries between an integrated HIV-1 provirus and adjoining cellular sequences (Figure 13).

Detailed restriction enzyme analysis identified two lambda phage clones (YU-3, YU-4) to exhibit extensive genomic rearrangements in the 5' halves of their genomes. Further characterization revealed that both clones contained extraneous HIV-1 LTR sequences in reverse orientation with respect to the remainder of the viral genome. To analyze more precisely the nature of these inverted LTR fragments and to elucidate the mechanisms by which they were generated, we determined their nucleotide sequences as well as the sequences of their insertion sites (Figure 14). Sequence analysis revealed that both HIV-1 genomes contained inverted LTR sequences within their pol genes. YU-4 contained an inverted LTR which was inserted in its entirety without additional deletions or rearrangements of the target sequence except for the presence of a 5bp direct repeat (AATAC) directly adjacent to the insertion site. The boundaries of the inverted LTR itself were identical to those of an integrated HIV-1 provirus (5'-TG...AC-3'). This fact, as well as the observed duplication of sequences (AATAC) normally present only once in this region of the HIV-1 genome, indicated that the inverted LTR in YU-4 was the result of an autointegration event. Inverted LTR sequences were also identified in the pol gene of YU-3. However, this inversion was associated with deletions involving both LTR and pol sequences. The LTR itself lacked 6bp on

its 3' and 3bp on its 5' end, while a major portion of the pol gene (1197bp) was also missing. No direct repeats were identified in the vicinity of the insertion site, and there were no additional deletions or alterations within the inverted LTR. Because of the absence of the characteristic target sequence duplication, the mechanisms responsible for the genomic rearrangements in YU-3 are less clear. Interestingly, genomic inversions in both YU-3 and YU-4 involved solely LTR sequences, and did not include adjacent viral sequences as has been observed in other retroviral systems. Also, there was no evidence for consensus sequences for target site recognition in regions adjacent to both inverted LTRs.

Current data regarding the genetic heterogeneity of HIV-1 have been derived from studies of virus cultures and from direct PCR amplification of primary, uncultured tissues (48,54-58). Important differences in results from these two approaches include a lesser degree of HIV-1 genetic variation *in vitro* and a surprisingly high proportion of defective viral genomes *in vivo* (58). The availability of both recombinant lambda and PCR derived molecular clones of HIV-1 from the same brain specimen allowed us to directly compare HIV-1 variability by these two approaches. Moreover, the PCR analyses served to independently confirm the origin and authenticity of the lambda phage clones. A 510bp fragment containing the V3 loop and adjacent envelope sequences was amplified by single round PCR, subcloned into M13, and subjected to nucleotide sequence analysis. A total of 12 independent M13 clones as well as eight lambda phage clones were sequenced in the same envelope region. An alignment of their sequences is depicted in Figure 15.

Comparing a total of 20 independent envelope sequences, we identified one predominant viral genotype as well as eleven minor viral variants. Importantly, the nucleotide sequence of the predominant genotype was identical both for lambda phage as well as PCR derived clones, thus proving unequivocally their common origin. Overall genotypic variability in 19 of 20 clones was

1.2% or less. Only one clone exhibited 8.2% nucleotide sequence differences. Lambda phage clones comprised a homogeneous group of viruses, with five clones having an identical nucleotide sequence and three others differing by only one or two point mutations (0.2% to 0.4% nucleotide sequence differences). PCR derived sequences varied more, both with respect to the number of nucleotide sequence substitutions between individual clones (0.2% to 8.2% nucleotide sequence differences) as well as with respect to the total number of distinguishable variants (8 of 12 versus 3 of 8 for lambda phage clones). In addition, only PCR derived sequences contained single base pair deletions as well as frequent G to A transitions. One PCR clone was characterized by a particularly large number of G to A changes which comprised 40 out of 42 total nucleotide substitutions.

All 12 distinguishable HIV-1 genotypes were also unique in their deduced amino acid sequence, indicating that the majority of nucleotide changes were non-silent in nature. Three of eight lambda phage clones differed from the predominant genotype by only one or two amino acid sequence changes, none of which interrupted the envelope open reading frame. Three of eight PCR derived clones varied from the predominant strain by a similar number of substitutions, while five other PCR clones contained frame shift mutations and/or in frame stop codons. This indicated that at least 42% of the PCR derived clones would correspond to defective viral genomes.

To investigate whether any of the four full-length HIV-1 genomes were replication competent, we isolated viral inserts from YU-2, YU-10, YU-21 and YU-32, self-ligated these inserts to generate viral genomes in nonpermuted orientation, and transfected them into Cos-1 cells. Primary transfectants were subsequently co-cultivated with PHA-stimulated normal donor PBMCs or immortalized T-cell lines in an attempt to transmit replication competent virus. Cultures were inspected daily for the appearance of virally-induced syncytia and monitored for retroviral activity

by p24 antigen capture and reverse transcriptase assays. Virus positive culture supernatants were filtered and transmitted to additional target cells to determine cell free infectivity and host cell range.

The results of these studies (Figure 16) demonstrated that only one of the four self-ligated inserts (YU-2) reproducibly yielded a productive viral infection in primary lymphocytes, monocytes and Molt4(clone8) cells. YU-2 derived virus was also cell-free transmissible to these cell types, but failed to replicate in SupT1 and CEMx174 cells. Cos-1 cells transfected with self-ligated YU-10 insert produced p24 transiently and formed syncytia after cocultivation. However, repeated attempts to establish a productive infection in primary PBMC or immortalized T-cell lines failed, suggesting a defect in one of the major viral proteins of YU-10. Self-ligated inserts of YU-21 and YU-32 failed to yield virus in all cell types tested including primary transfected Cos-1 cells.

To improve the transfection efficiency, we reconstructed two of the four full-length lambda phage clones (YU-2 and YU-10) to generate viral genomes in nonpermuted orientation. This was achieved by subcloning a SalI/EcoRI fragment (containing the 5' half of the virus) and a EcoRI/SphI fragment (containing the 3' half of the virus) into the same plasmid vector (Figure 13). Subsequent transfection experiments confirmed the results utilizing self-ligated lambda phage inserts. pYU-2 derived virus replicated well in primary lymphocytes and monocytes and induced syncytia when cocultivated with Molt4(clone8) cells (Figure 16). Electron microscopy demonstrated normal particle morphology, and Western blot analysis confirmed the presence and appropriate size of all virus specific proteins. Importantly, pYU-2 derived virus infected primary macrophages and replicated to high titers, exhibiting growth characteristics similar to those previously identified for macrophage-tropic HIV-1 strains (48). Transfection of pYU-10 failed to result in a productive viral infection even in the nonpermuted plasmid construct. This strongly

suggested that YU-10 was replication defective, although certain viral genes, including the gag and the env gene, appeared to be functional since transient p24 production and syncytium formation was observed. Thus, the results of this analysis revealed for the first time that replication competent HIV-1 genomes, complex mixtures of defective viral forms, and chromosomally integrated provirus persist *in vivo*. In addition, the brain-derived viral clones were expected to prove valuable for future studies of macrophage and neurotropism, as well as for the analysis of other viral properties which are subject to *in vitro* selection pressures.

6. <u>A Molecular Clone of HIV-1 Tropic and Cytopathic for Human and Chimpanzee Lymphocytes.</u>

The molecular cloning of complete proviral genomes made possible the specific genetic identification of the human immunodeficiency virus-type 1 (HIV-1) (59). Proviral clones also provided the necessary molecular reagents for identifying and studying the function of tat, rev, vif, vpr, vpu and nef genes, which along with gag, pol, and env, characterize this and other primate immunodeficiency viruses (60). More recently, proviral clones have been used to characterize viral determinants of cell tropism, cytopathicity, and virulence in cultured cells and in animal models (reviewed in 61). Many studies have utilized chimeric HIV-1 proviruses as the genetic background in which to examine the function of individual genes as well as more complex viral properties such as entry and replication (60,61). Because the products of more than one gene, or even different parts of a single gene, can affect the same phenotypic characteristics (e.g., vif and env affect entry; vpu and env affect syncytium induction; tat and env affect replication kinetics; env-gp120 and env-gp41 affect envelope conformation and function (21), it may be advantageous in some circumstances to examine the molecular properties of HIV-1 using proviruses cloned as single (complete) interactive genetic units. Interestingly, only four replication competent and fully

sequenced HIV-1 proviruses, cloned as single interactive genetic units, have been reported (HXB-2d, ARV-2, NDK, and YU-2) (61). None of these has been developed in an HIV-1 AIDS animal model, and no molecularly cloned HIV-1 strains have been described which replicate in chimpanzee cells in vitro or in vivo.

In an attempt to identify HIV-1 proviral clones for analysis of viral replication in human and chimpanzee cell culture as well as in animal model systems, we first examined the replicative properties of a series of HIV-1 isolates in cultured primary human and chimpanzee PBMCs. Both preexisting and newly derived HIV-1 strains were studied, including virus obtained by transfection of proviral DNA cloned from uncultured human brain (YU-2; see above) and from cultured PBMCs of an individual with acute HIV-1 infection (WEAU 1. 60; see above); prototypic virus isolates LAV-1b (a chimpanzee-passaged strain of LAV-1); ARV-2, and NDK; and a virus isolate designated BC that was obtained by short-term co-culture of Hut 78 cells with PHA-stimulated PBMCs of an individual in the terminal stages of AIDS (61,62). The replicative characteristics of isolates IIIb, MN, and RF in chimpanzee lymphocytes were previously reported (63,64). All viruses replicated efficiently in human cells, but only LAV-1b, NDK, and BC replicated efficiently in chimpanzee cells. These experiments were repeated in PBMCs from three different chimpanzees with similar results, indicating that these three virus isolates, compared to the others studied, reproducibly showed preferential replication in chimpanzee cells (data not shown).

Because of its replicative and cytopathic activity in primary human and chimpanzee lymphocytes and in immortalized human T-cell lines, the BC isolate was selected for further molecular and biological characterization. Total cellular DNA from the Hut 78/BC cell culture was analyzed by restriction enzyme digestion and Southern hybridization (Fig. 17A), and a recombinant lambda phage library was prepared by methods previously described (48), using the cloning vector

λGEM-12 and the viral non-cutter EcoRI. Three full-length proviral clones (λSG3, λSG15, and λSG39) were identified, one of which (λSG3) was replication competent and produced virions whose replicative and cytopathic properties were equivalent to those of the parental BC isolate. The SG3 provirus, including flanking cellular sequences (total length, 16.0 Kb), was subcloned into the Eco RI site of pTZ 19U (United States Biochemicals). This recombinant plasmid, in turn, was digested with Bam HI in order to remove a 4.4 Kb fragment of flanking cellular DNA, resulting in the clone pSG3.1 shown in Fig. 17B. The restriction endonuclease cleavage pattern of pSG3.1 was identical to that of the predominant proviral form within the parental BC isolate (Fig. 17A).

Biological characterization of the SG3 virus strain was performed using virus stocks generated by calcium phosphate-mediated transfection of pSG3.1 DNA in Cos-1 cells (2). Fig. 18 illustrates the protein composition (A), cytopathic activity (B), and replication characteristics (C) of SG3 in primary human cells and immortalized T-cell lines. SG3 was similar to prototype HIV-1 in structural protein composition and similar to T-cell line-adapted viruses in its ability to replicate in primary and immortalized lymphocytes but not in monocyte-macrophages. SG3 exhibited the most striking cytopathic activity in T-cell lines of any virus strain that we have studied, leading to rapid and extensive syncytium formation and cell death.

SG3, LAV-1b, and NDK were analyzed under identical experimental conditions for their ability to replicate in primary human and chimpanzee PBMCs. Ten million PHA-stimulated normal human and chimpanzee PBMCs were infected with identically prepared virus stocks (1 x 104 TCID50) that had been titered on human PBMCs. As shown in Fig. 19, SG3 replicated faster and to substantially higher levels (705,205 cpm versus 10,950 cpm RT activity, day 7) in chimpanzee cells than in human cells. In three separate experiments using PBMCs from different chimpanzees and from different human donors, supernatant RT activity at day 7 averaged 1,269,683 cpm for

SG3-infected chimpanzee cells compared with 45,228 cpm for SG3-infected human cells. Conversely, LAV-1b and NDK replicated more rapidly and to equivalent titers in human versus chimpanzee cells.

We also determined the replicative and cytopathic activity of SG3 and LAV-1b in purified chimpanzee CD4+ lymphocytes (Fig. 20). Again, SG3 replicated more rapidly and to higher titers than did LAV-1b (2,804,558 cpm versus 354,433 cpm RT, day 6). SG3 and LAV-1b, but not WEAU 1.60, led to significant killing of CD4+ lymphocytes compared to mock-infected cultures.

Because of the potential utility of the pSG3.1 proviral clone for molecular studies of HIV-1 gene function in vitro and in vivo, we determined its complete nucleotide sequence and genome organization (Fig. 21A). From the beginning of the R region of the 5' LTR (corresponding to the mRNA start site) to the polyadenylation site in the terminus of R in the 3' LTR, the provirus measured 9,168 nucleotides and consisted of 5'-LTR-gag-pol-vif-vpr-tat-rev-env-nef-LTR-3'. The regulatory and structural genes of SG3 were generally typical of other HIV-1 strains in their primary sequences and identifiable functional motifs, with certain exceptions. The vpu gene, normally 243 nucleotides in length (60), was disrupted after 70 nucleotides by a 23-nucleotide deletion that resulted in translational frameshift and truncation of the open reading frame (Fig. 21B). Polymerase chain reaction amplification of this region from the parental Hut 78/BC cell line confirmed the presence of the vpu deletion in greater than 90% of the proviral molecules present. The env gene, although similar to prototype HIV-1 strains in length (2577 nucleotides), overall content of cysteine and potential N-linked glycosylation residues (21 and 27, respectively), and presence of typical hypervariable (V1-V5) regions (60), was interesting in two respects. First, the V3 variable region (CTRPNKKTRKRITTGPGRVYYTTGEIVGDIRQAHC) contained two adjacent basic residues (lys-lys) at positions 6 and 7 within the loop which is atypical for HIV-1.

Secondly, the overall V3 peptide charge at pH7.0, calculated by the CHARGEPRO program from PCGENE (IntelliGenetics), was highly positive (+5.83). A strongly positive V3 region charge has previously been associated with T-cell tropism and syncytium induction in other HIV-1 strains (65) and this property may be contributing to the biological phenotype of SG3.

Phylogenetic analyses were performed to ascertain the relationship of SG3 to other HIV-1 strains, and to assess whether SG3 might be a recombinant virus. First, the predicted protein sequences of the four major genes (gag, pol, env, and nef) were compared with those for a number of strains of African (U455, ELI, NDK, OYI) and non-African (LAI, MN, JRFL, JRCSF, SF2, HAN, RF, YU-2) origin for which full-length sequences have been determined, using the neighbor-joining algorithm in CLUSTALV and taking SIVCPZ as an outgroup. Previous phylogenetic analyses have indicated that all North American (and European) strains of HIV-1 form a cluster quite separate from strains of African origin, except for OYI which falls within the North American cluster. For each of these four protein sequences, SG3 clustered within the North American subgroup, although it was not particularly closely related to any other strain (data not shown). Only minor differences in branching order were found among the four trees, and these differences were not statistically significant as assessed by bootstrap analyses. Thus, there was no evidence for intergenic recombination during the recent evolution of SG3. Second, the phylogenetic relationship of SG3 to a large number of HIV-1 strains for which env gene sequences have been determined was investigated by DNA sequence comparisons (Fig. 22). Again, SG3 was seen to fall within the North American subgroup, with no significant similarity to any other isolate within that cluster. Third, the V3 sequence of SG3 was examined specifically for its relationship to SIVCPZ and other HIV-1 strains. The V3 sequence of SG3 differed in at least 4 residues from every other reported HIV-1 strain. There were 20 differences between SG3 and SIVCPZ and 8

differences between SG3 and the North American V3 consensus sequence (66), only one of which was shared with SIV^{CPZ} (the Val at position 27, which was also found in HAN). Thus in none of our phylogenetic analyses was SG3 seen to be more similar, than any other HIV-1 strain, to SIV_{CPZ}.

In this report, we describe the derivation, genome organization, phylogenetic identity, and biologic properties of an HIV-1 proviral clone (SG3) that replicates efficiently in both chimpanzee and human cells. It should be emphasized that SG3 was molecularly cloned as a single interactive proviral unit and not as a chimeric construction representing different viral genomes. This may be important in interpreting certain viral properties since the functional activity of one part of the genome is often influenced by other regions of the same or a different gene. In the case of SG3, for example, the deletion of vpu may well contribute to this virus's ability to replicate efficiently in chimpanzee lymphocytes and to its pronounced syncytium-forming properties in human T-cell lines. Terwilliger et al (67) and Klimkait et al (68) have both shown that HIV-1 viruses defective in vpu exhibit enhanced cytopathicity and impaired virus release but the existence and biological importance of such virus strains in vivo is unknown.

To our knowledge, SG3 is the only molecularly-derived strain of HIV-1 that has been shown to replicate in chimpanzee PBMCs and the only HIV-1 strain of any derivation to exhibit preferential replication in chimpanzee PBMCs compared with human PBMCs. We determined directly that SG3 could replicate efficiently in CD4-enriched chimpanzee lymphocytes, but we did not examine its replicative properties in chimpanzee monocytes. Such studies are underway since it has been suggested that monocyte-macrophage tropism might correlate with enhanced viral pathogenicity in vivo (69). In this regard, pilot studies have shown that SG3 can lead to productive and persistent infection of a chimpanzee following cell-free intravenous virus inoculation (P. Fultz,

unpublished data) and of SCID-hu mice following intrathymic inoculation (L. Rabin and J. M. McCune, personal communication). Whether or not SG3 will cause CD4 lymphocyte depletion or disease in these animals will require further study.

Previously, Watanabe and co-workers (64) showed that a chimpanzee-passaged HIV-1 strain (LAV-1b) could replicate efficiently and cytopathically in chimpanzee lymphocytes in vitro. We confirmed this in our studies but found that SG3 replicated even more rapidly and to higher titers compared with LAV-1b. Camerini and Seed (70) reported that cells expressing the chimpanzee CD4 molecule, while susceptible to HIV-1 infection and replication, are relatively resistant to viral envelope-mediated cell-cell fusion compared with cells expressing the human CD4 gene. This biological difference was found to result from a glutamine for glutamic acid replacement at amino acid position 87 of the chimpanzee CD4 molecule. Our findings are not necessarily inconsistent with those of Camerini and Seed since it is now known that a number of viral factors as well as CD4 can influence cell tropism and syncytium induction. Such factors include gp120 affinity for CD4, envelope determinants that lie within and outside the V3 loop, and viral products such as vpu that alter the expression of other viral proteins such as the envelope.

The cytopathic and replicative activity of SG3 in vitro in primary chimpanzee and human lymphocytes and in immortalized human T-cell lines, as well as its ability to productively infect chimpanzees and SCID-hu mice in vivo, make it a promising reagent for analyzing molecular determinants of viral pathogenesis. We are currently conducting chimeric virus studies utilizing SG3 and two other biologically interesting HIV-1 proviral clones, YU-2 and WEAU 1. 60, to examine viral determinants responsible for cell tropism, cytopathicity, and virulence in cultured cells and in chimpanzee and SCID-hu mouse model systems. In addition, we are exploring the potential for replication and pathogenesis of SG3 in the newly described pig-tailed macaque

HIV-1 animal model system (71).

7. <u>Use of PCR amplification and "universal" (consensus) oligonucleotide primer sequences to identify, clone, and characterize divergent or novel human immunodeficiency viruses in high risk populations</u>.

Current understanding of the biology and origins of human immunodeficiency virus type 2 (HIV-2) has derived from studies of cultured isolates from urban populations experiencing epidemic infection and disease (72-79). To test the hypothesis that such isolates might represent only a subset of a larger, genetically more diverse group of viruses, we used nested polymerase chain reaction (PCR) to characterize HIV-2 sequences in uncultured blood mononuclear cells of two healthy Liberian agricultural workers (F0784; 2238) from whom virus isolation was repeatedly unsuccessful and from a culture-positive symptomatic urban dweller (7312A). Previously, we had conducted a seroepidemiological survey in Liberia, West Africa to estimate the prevalence of HIV-1 and HIV-2. Serum samples were collected from 372 healthy adults living in remote villages of northern Liberia, 944 rubber plantation workers attending outpatient clinics in rural areas of central Liberia, and 366 adults from the capital city of Monrovia and surrounding urban areas. Three individuals were seropositive for HIV-1. Five other individuals, all healthy inhabitants of rural villages, were seropositive for HIV-2. Two of these individuals (F0784 and 2238) could be relocated for additional serologic testing, virus culture, and PCR analysis. Both were male rubber plantation workers, ages 46 and 47, with normal physical examinations, no history of sexually transmitted disease, chronic illness, blood transfusion, or homosexuality. For comparison, a third HIV-2 seropositive but symptomatic man from Abidjan, Côte d'Ivoire was also studied. He was 32 years old, had lymphadenopathy, cutaneous anergy and recurrent skin abscesses, and admitted to frequent sexual encounters with urban prostitutes. Blood specimens from all three subjects were

obtained on two separate occasions and processed for virus isolation and PCR analysis (80,81). Mononuclear cells (PBMCs) were cultured alone, in combination with normal donor lymphocytes and macrophages, and with immortalized T-cell lines (Molt4 clone8, CEMx174, H9, SupT1). HIV-2 was successfully isolated from subject 7312A on each of two occasions but not from subjects F0784 and 2238, despite excellent cell growth and viability.

To determine the genetic identity of viral sequences in subjects FO784, 2238, and 7312A, we employed a highly-sensitive nested PCR technique to amplify viral sequences directly from uncultured PBMC DNA (80,81). Using primer pairs designed according to HIV-2/SIV_{MAC}/SIV_{SM} consensus sequences (82), 708bp *pol* and 453bp *env* fragments were amplified (Figure 23) and sequences corresponding to a total 34,770 nucleotides determined This analysis revealed viral mixtures, or quasispecies, of varying complexity for each subject (Figure 24). FO784 harbored the largest number of variants, most of which represented defective (prematurely terminated) viral genomes which resulted from G to A hypermutation. Such G to A changes, which were found in two different genes (*pol* and *env*) and in blood samples obtained four months apart, accounted for 66% to 87% of all nucleotide substitutions in FO784 and resulted in sequence differences among individual clones as high as 11.5%. *Env* and *pol* regions from 2238 and 7312A exhibited much less intrastrain variability (< 0.3%) and no G to A hypermutation, which together with our previous studies of SIV_{AGM} (80), indicated that *Taq* polymerase or sequencing errors did not contribute significantly to the G to A changes observed in FO784.

To determine the phylogenetic relationships of HIV-2_{FO784}, HIV-2_{2238}, HIV-2_{7312A}, and other HIV-2/SIV_{SM}/SIV_{MAC} strains, we constructed evolutionary trees for their *pol* and *env* sequences using both the neighbor-joining approach (84,85) (a reliable method which allows for unequal rates

of evolution) and the maximum parsimony algorithm of PAUP (86). In both *pol* (Figure 25A) and *env* (Figure 25B) regions, seven previously reported prototype isolates of HIV-2 clustered in a closely related group. In contrast, the three strains reported here branched quite differently. HIV-2_{2238} was most closely related to a single divergent HIV-2 isolate, HIV-2_{D205} (78), although these two viruses differed from each other to a greater extent than was typical for prototypic HIV-2 strains. HIV-2_{7312A} appeared to have a mosaic genome: its *pol* sequence was most closely related to HIV-2_{D205} (Figure 25A), yet its *env* sequence clustered with the prototypic HIV-2 isolates (Figure 25B). These discordant relationships for HIV-2_{7312A} *pol* and *env* regions were strongly supported by bootstrap analyses (87) and most likely reflect recombination between phylogenetically divergent strains.

The most striking finding from the phylogenetic analysis was that HIV-2_{F0784} *pol* and *env* sequences clustered with the simian viruses, SIV_{SM} and SIV_{MAC}, rather than with other HIV-2 strains. This relationship was found in the majority, though less than 95%, of bootstrap resamples, and so to substantiate the phylogenetic position of HIV-2_{F0784}, we amplified and analyzed two additional regions of its genome: a much larger 1972bp *pol* fragment encoding reverse transcriptase (RT) and a 717bp *nef*/LTR fragment (Figure 23). Phylogenetic analysis of the RT fragment (Figure 25C) demonstrated clustering of HIV-2_{F0784} with the SIV lineage in more than 99.9% of bootstrap resamples. The *nef*/LTR region of HIV-2_{F0784}, chosen for analysis because it encompasses a 40-44bp "signature" sequence that is present in all published clones of HIV-2 (including HIV-2_{D205}) but absent from all SIV_{SM}/SIV_{MAC} genomes (82), lacked this sequence (Figure 26) which therefore no longer distinguishes between viruses that infect man and monkeys. Overall, our phylogenetic analyses thus indicated that HIV-2 in man and SIV in mangabeys and captive macaques represent

genetically-diverse members of a *single* group of viruses.

For several reasons, we consider it most likely that the natural reservoir for the entire group of HIV-2-related viruses is the sooty mangabey and that HIV-2 infection of man represents a zoonosis (88). First, SIV_{SM} is a pathogenic in sooty mangabeys (89,90) but is pathogenic in man and macaques (91-93), as is the case for many zoonotic infections which cause less severe or even no disease in their natural hosts (88). Second, the natural habitat of the sooty mangabey generally coincides with the geographic pattern of HIV-2 endemicity in West Africa (90). Third, close contact between mangabeys and man and between mangabeys and captive macaques is well-documented (90,93,94). These observations, together with phylogenetic data showing a close overall genetic relationship between HIV-2 and SIV_{SM}(94) yet deep and roughly equidistantly branching lineages for the different subgroups (Figure 25), argue for multiple introductions of genetically-diverse SIV_{SM} viruses into different human and simian populations.

It is notable that most previously studied isolates of HIV-2 were obtained by virus culture from individuals who were generally symptomatic and resided in areas where HIV-2 was spreading epidemically (72-79). In contrast, HIV-2_{FO784} and HIV-2_{2238} could not be cultured (despite several attempts) from healthy inhabitants of rural areas where HIV seroprevalence was low and AIDS had not been recognized clinically. This raises the possibility that certain naturally-occurring strains of HIV-2 may replicate less efficiently and cause less virulent infection. In this regard, it is of interest that the majority of HIV-2 sequences from subject FO784 contained multiply-defective genomes and an inordinate number of G to A substitutions. Biased G to A hypermutation has previously been described for HIV-1 (95), but not to the extent found in HIV-2_{FO784}. Studies are underway to determine the molecular basis for G to A hypermutation and whether, in the extreme case, it could result in attenuated or even abortive viral infection.

Finally, a recent suggestion (96) that SIV_{SM} may have been accidentally transmitted to man by inoculation with infected monkey blood probably cannot explain the diversity now recognized for HIV-2. Results of our studies suggest that viruses from feral monkey populations and humans living in remote areas of Africa should be targeted in a search for the origins of simian and human immunodeficiency viruses and the events leading to their recent epidemic spread.

Conclusions

The results of these studies have brought clearer insight into virologic processes underlying HIV-1 pathogenesis. The combination of quantitative plasma virus cultures, p24 antigen determinations, and most recently, direct measurements of virion-associated RNA by competitive PCR (QC-PCR) (32) demonstrated conclusively that HIV-1 replication is continuous throughout all stages of infection even during relatively asymptomatic clinical periods. These findings have been corroborated independently by recent studies by Fauci and colleagues (30) and by earlier studies by Ho (19) and Michael (35) and their co-workers. The levels of circulating virus are related to clinical stage and CD4 counts,and antiretroviral therapy with RT inhibitors leads to a 10-100 fold reduction in circulating virus (32). However, the development of viral resistance often leads to a return of viremia titers to pretreatment levels (46). These results thus suggest a direct virologic basis for HIV-1 pathogenesis and provide a practical approach for assessing antiretroviral therapy *in vivo* in all patients, at all stages of disease, and at all CD4 levels.

By cloning full-length, replication-competent provirus from cultured and uncultured patient tissue specimens (36,48,61), and by determining their nucleotide sequences and biological properties, we have also extended our insights into molecular determinants of viral tropism and pathogenesis *in vivo*. In this report and in the accompanying manuscripts, we describe the first

full-length HIV-1 proviral clones obtained from *uncultured* human tissue (36,48). These clones include replication-competent as well as minimally defective genomes whose corresponding viruses exhibit striking macrophage tropism. These virologic reagents in turn allowed us to identify envelope regions of naturally-occurring primary viruses responsible for macrophage and T-cell tropism (97) and should allow the delineation of syncytium-inducing determinants as well. Importantly, these same clones can be explored as potential proviral vectors for the development of HIV-1 vaccines including a candidate live-attenuated virus vaccine (98). In addition, the above-described studies demonstrated for the first time remarkably high ratios of defective (non-culturable) to infectious virus circulating in human plasma (32). The role of these different viral forms in HIV-1 pathogenesis will be an important area of future study.

Finally, our studies have led to the identification of HIV-2 strains in West African people which are phylogenetically indistinguishable from SIV strains infecting feral sooty mangabey monkeys from the same region (99). The studies, together with earlier work noting the similarities in genomic organization between these two virus groups (94), provided convincing evidence for cross-species transmission of virus from monkey to man as the origin of HIV-2. A similar scenario is now believed to be likely as the origin of HIV-1. These findings are important not only to explain lentiviral phylogeny but also because they are informative regarding basic mechanisms involved in HIV pathogenesis. The studies described in this report thus bring us a step closer to a mechanistic understanding of HIV natural history and pathogenesis and a step closer to the development of effective treatments and vaccines.

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APPENDIX A: TABLES AND FIGURES

Table 1. Human immunodeficiency virus (HIV) type 1 plasma titers and clinical characteristics in adults.

Virus titer, patient	Date of evaluation (1989)	CDC stage	CD4 cells/mm^3	HIV p24 Antibody titer	HIV p24 Antigen (pg/ml)	Therapy
No growth (negative)						
EDRI-0025	01/19	II	1904	88	26	D
RIPH-0179	08/24	II	1080	0	0	N
MDJO-0252	07/20	II	996	QNS	0	N
GICL-0061	05/10	IVC2	798	0	37	N
ATKA-0381	12/28	II	760	414	0	N
HOJU-0143	06/28	II	720	14,763	0	N
WHMA-0128	09/14	II	713	QNS	0	Z
VAST-0181	07/20	IVC2	710	1	0	Z
MAMA-0341	10/25	II	705	64	0	N
ADFR-0194	05/10	II	703	387	0	N
BECH-0171	03/09	II	678	596	0	N
MALE-0264	07/26	III	644	3138	0	N
ROJO-0331	11/29	II	640	574	0	N
BAMA-0037	03/22	II	624	0	0	N
ADDI-0101	03/15	II	616	1709	0	N
ALFR-0229	08/23	IVC2	615	390,625	0	N
CADA-0309	11/08	IVC2	595	872	0	N
KEBO-0042	06/21	II	594	20	0	N
ERBR-0339	10/25	II	589	6	0	N
GIRO-0137	10/11	II	585	290	0	N
WIMI-0366	10/31	II	572	9092	0	N
CADA-0024	05/17	IVC2	529	1	0	N
SIWE-0201	05/03	II	528	22	0	N
BRST-0214	05/17	III	522	98	0	N
DALE-0069	12/21	III	520	1	72	N
COBI-0232	05/31	III	504	8243	0	N
PORA-0355	11/01	III	483	0	32	N
FOMA-0117	03/08	IVC2	476	0	10	N
HUAL-0211	05/03	III	468	758	0	N
SCRI-0023	03/01	II	462	259	0	N
AXBO-0085	02/16	IVC2	441	0	0	N
SMMA-0222	05/17	IVC2	440	900	0	N
LIWE-0187	09/27	IVC2	435	17	0	N
JAJO-0083	05/24	II	432	41,311	0	N
RYJE-0273	08/23	III	420	0	36	N
KOFL-0311	11/15	III	396	359	0	N
CASH-0292	10/04	IVC2	378	1	6	N
JUJA-0156	05/18	III	360	2889	0	H
HATI-0385	12/28	IVC2	341	6402	0	N
WAFR-0077	10/04	IVC2	312	65	0	N
NADA-0234	10/03	III	311	46	0	N
PAAL-0266	09/06	IVC2	273	2506	0	N
HOKE-336	11/29	IVC2	240	0	62	N
MMRA-0007	01/26	IVC1	239	51	51	Z
SATI-0088	04/04	IVC2	234	15,135	0	N
STDA-0183	07/27	IVC2	234	390,347	0	Z
PLJO-0293	10/18	IVC2	228	270	0	N
REJA-0225	07/06	IVC2	220	358	0	N
CHPA-0351	11/21	IVC2	220	1	0	N
DAAD-0212	09/06	IVC2	78	1	9	N
10^1 TCID/ml						
COWI-0019	03/29	IVC1	5	16	0	N

Table 1. *Continued.*

Virus titer, patient	Date of evaluation (1989)	CDC stage	CD4 cells/mm^3	HIV p24 Antibody titer	HIV p24 Antigen (pg/ml)	Therapy
10^2 TCID/ml						
ISBU-0089	03/23	IVAB	3	0	31	N
GIRO-0005	02/02	IVC1	9	0	14	Z
REMI-0022	03/15	IVD	11	29	0	N
TIMI-0018	03/02	IVC1	14	0	29	N
HADO-0110	05/11	IVC2	20	5	21	N
GOJU-0183	03/29	IVC1	23	500	0	N
LUDA-0153	03/22	IVC1	71	0	74	N
WERI-0233	06/28	IVC2	286	0	30	N
MCSE-0176	05/24	IVC2	360	1	960	N
10^3 TCID/ml						
NAPH-0073	03/16	IVC1	14	0	70	N
LAJO-0060	04/04	IVC1	19	108	3	Z
WIAL-0014	01/26	IVC1A	20	0	40	N
BIJA-0205	05/11	IVC1	32	7	770	N
10^4 TCID/ml						
DEDA-0006	03/15	IVC1	57	1	0	N
SMDO-0157	03/02	IVC2	282	1	250	N
10^5 TCID/ml						
DODO-0116	01/19	IVC1	50	0	98	N
10^6 TCID/ml						
JOJI-0070*	02/10	IVC1	4	0	250	N

NOTE. Virus titer = quantitative plasma virus titer log base 10 (TCID/ml of plasma); CDC = Centers for Disease Control (stage II, asymptomatic; III and IVC2, AIDS-related complex; IVAB, IVC1, and IVD = AIDS); therapy = antiviral therapy at time of evaluation (N = none, Z = zidovudine, H = hypericin, D = ditiocarb). QNS = quantity not sufficient for analysis.

Table 2. Human immunodeficiency virus (HIV) type 1 plasma titers and clinical characteristics in children.

Virus titer, patient	Date of evaluation (1989)	Age at evaluation	Risk	CDC stage	CD4 cell/mm^3[a]	HIV p24 Antibody titer	HIV p24 Antigen (pg/ml)
No growth (negative)							
WIBB-C013	10/31	1 day	P	P0	1025	0	0
CADE-C009	09/20	8 months	P	P0	536	0	0
JERO-C012	10/18	18 months	P	P0	2848	0	0
MIDA-C014	12/06	18 months	P	P0	904	0	0
BRRO-C011	10/11	2 years	P	P0	1355	0	0
CACH-C016	12/20	9 years	H	P2A	359	8096	0
LACL-C008	10/18	11 years	H	P1B	407	3970	1.0
WOME-C242	06/28	14 years	H	IVC2	228	1	28.6
STDA-C007	07/13	5.5 years	T	P2F	180	0	71.2
10^1 TCID/ml							
GRAL-C015	12/13	3 years	P	P2D2F	1628	9	0
10^3 TCID/ml							
HUJO-C005[b]	08/02	6 weeks	P	P1A	2227	822	0
MUWI-C003	05/25	6 months	P	P2D1	150	QNS	345.0
MOBG-C004	06/21	10 months	P	P2AF	572	QNS	6.4
WHRO-C002	06/21	14 months	P	P2ABD3	42	0	54.0

NOTE. Virus titer = quantitative plasma virus titer log base 10 (TCID/ml of plasma); risk = mode of infection or exposure (*P* = perinatal, T = transfusion recipient, H = hemophiliac); CDC = Centers for Disease control classification of HIV-1 infection in children <13 years [22]; therapy = antiviral therapy at time of evaluation; QNS = quantity not sufficient for analysis.

[a] Normal ranges (mean ± 2 SD) for CD4-lymphocyte numbers in children 0–6 months, 3166 ± 2100/mm^3 (n = 106); 6–12 months, 2828 ± 2300 (n = 28); 12–24 months, 2306 ± 1890 (n = 46); 24–74 months, 1688 ± 1190 (n = 29); adults, 1027 ± 770 (n = 327).

[b] Treated with zidovudine. All other children were untreated.

Table 3. Sequential and replicated plasma human immunodeficiency virus (HIV) titer determinations.

Patient	Date of evaluation (1989)	Normal PBL donor	HIV p24 antigen level (pg/ml)	Plasma virus titer
BIJA 0205	05/11	D1	770	3
	05/23	D2	75	2
	05/23	D3		3
	05/23	D4a		3
	05/23	D4b		3
	05/23	D4c		3
	12/14	D5	293	4
DODO 0116	02/01	D6	49	5
	02/08	D7	98	3
SMDO 0157	03/02	D8	250	4
	08/15	D9	998	4
DEDA 0006	03/15	D10	0	4
	05/31	D11	0	3
WHRO C002	06/21	D12	54	3
	11/15	D13	165	2
NAPH 0073	03/16	D14	70	3
	07/11	D15	27	2
	07/11	D16		1
	07/11	D17a		2
	07/11	D17b		2
	07/11	D17c		2

NOTE. Normal peripheral blood lymphocytes (PBL) D1–D17 are from 17 different normal donors; D4a, D4b, D4c and D17a, D17b, and D17c are PBL from individual donors that were cultured in triplicate sets of wells. Plasma virus titer = titer of HIV type 1 cultured from plasma (log base 10: TCID/ml of plasma). No subject except NAPH received antiviral therapy.

TABLE 4: Signs and symptoms of acute HIV-1 infection.

SIGN OR SYMPTOM	FREQUENCY (%)
Fever	97
Adenopathy	77
Pharyngitis	73
Rash	70
Myalgia or arthralgia	58
Thrombocytopenia	51
Leukopenia	38
Diarrhea	33
Headache	30
Elevated serum aminotransferase levels	23
Nausea or vomiting	20
Hepatosplenomegaly	17
Oral thrush	10
Encephalopathy	8
Neuropathy	8

* As identified in a review of 139 reported cases (ref 2). Also reported, though less frequently, were esophageal ulceration, esophageal candidiasis, vasculitis, nephritis, rhabdomyolysis with acute renal failure, hypoxemia, and fatal aplastic anemia.

Table 5 Virologic and clinical summary for 66 consecutively studied HIV-1–infected patients.

Patient ID	CD4+ cells* (cells/mm³)	HIV RNA† (copies/ml)	HIV p24 Ag ICD‡ (pg/ml)	HIV p24 Ag Reg§ (pg/ml)	Plasma‖ culture (TCID/ml)
CDC stage I¶					
HOBR 0961	920	2,617,400	386	660	125
SUMA 0874	853	1,485,000	892	1,250	625
BORI 0637	817	2,398,600	218	258	1#
INME 0632	739	2,427,900	275	360	1,000
WEAU 0575	358	355,400	258	329	0**
FASH 1057	262	21,783,600	5,072	5,406	10,000
CDC stage II or III					
RIPH 0179	1080	40,800	39	0	0
HIDO 1099	823	36,000	0	0	0
ATKA 0381	760	9,400	0	0	0
HOJU 0143	720	38,900	0	0	0
MAMA 0341	705	113,400	31	0	0
ADFR 0194	703	24,200	0	0	0
TIMI 0852††	678	100‡‡	0	0	0
BECH 0171	678	13,800	8	7	0
MALE 0264	644	198,800	0	0	0
ROJO 0331	640	78,700	0	0	0
BELI 1233	627	4,400	0	0	0
HAJO 0940††	624	13,800	0	0	0
ADDI 0101	616	128,300	0	0	0
FOMA 0784††	521	34,400	0	0	0
JUJA 0156	458	586,100	0	0	5
WATH 0272††	350	67,200	0	0	0
BAMA 0037	323	91,000	154	53	0
WAJO 1286	281	46,100	183	35	0
BAST 0514††	270	9,400	0	0	0
STMI 0862	257	92,900	574	350	5
STBO 1287	231	8,600	0	0	0
SLMI 0843	197	84,900	0	0	0
CDC stage IVC2					
ALFR 0229	615	91,800	0	0	0
DOBE 0859	533	114,500	0	0	0
CALI 0950††	456	40,800	35	5	0
ARLA 0846	435	49,100	0	0	0
WOAL 0263††	425	32,800	0	0	0
GADA 1162††	424	192,500	0	0	0
MCSE 0176	360	341,000	5,000	1,070	100
CHJI 0774	332	33,300	0	0	0
EDWI 0817	267	73,100	0	0	0
GRJO 0849	243	94,700	0	0	25
MIWI 1278	236	173,600	79	0	0
SMDO 0157††	141	2,200,000	740	284	3,125
HEMI 0562	117	223,000	123	101	125
DAJO 0306	109	36,900	361	120	0
SMST 1012	95	738,900	765	90	0
DUSE 1021	82	655,500	179	119	5
WHWI 1106	67	104,500	221	27	25
NOWR 1192	54	469,900	209	20	3,125
MIGE 1132††	29	191,800	0	0	0
DABE 0775	27	448,200	173	153	5
EMJA 0809	21	625,900	187	226	25
CLRA 0703	10	687,900	396	40	25
LARO 0833	7	479,300	823	238	5
CDC stage IVC1					
DEDA 0006	57	1,309,000	233	14	10,000
VAST 0181††	56	664,500	450	25	0
DODO 0116	50	1,667,000	357	385	100,000
SZHO 1173††	37	808,300	631	195	625
MILA 0284††	32	815,100	0	0	0
BIJA 0205	32	4,744,000	1,920	1,050	10,000
NAPH 0073	14	1,804,000	480	480	1,000
TIMI 0018	14	3,445,000	330	390	100
WATI 0855	7	9,300,000	606	302	625
MCMI 0063	7	232,000	221	12	625
LENA 1029	<5	2,500,000	53	0	125
FARO 1042	<5	4,800,000	199	204	625
RUTH 1145	<5	424,800	230	143	125
YOAL 0522	<5	2,600,000	205	5	125
JOJI 0070	<5	1,606,000	2,460	885	100,000

*CD4+ cells determined by flow cytometry within 6 weeks of sampling for virologic assessments. †HIV RNA copy number per milliliter of plasma, as determined by QC-PCR (*13, 17*). ‡Immune complex–dissociated HIV p24 antigen (*19*). §HIV p24 antigen (*19*). ‖Tissue culture infectious doses of virus per milliliter of plasma (*18*). ¶Centers for Disease Control classification system for staging of HIV-1 infection (*2*). #Three days previously, plasma cultures were positive at 10 TCID/ml, with HIV-1 RNA level of 1,350,600 copies/ml. **Eight days previously, plasma cultures were positive at 1,000 TCID/ml, with HIV-1 RNA level of 216,400 copies/ml (Fig. 2). ††AZT or dideoxyinosine therapy at time of study. ‡‡Extrapolated value.

Table 6 AZT treatment: Virologic and clinical data summary. Table shows values for patients before and after treatment with AZT for the indicated periods. ND, not determined.

Patient ID	HIV RNA (copies/ml)	HIV p24 Ag*		Plasma culture (TCID/ml)	AZT	Time on treatment (weeks)
		ICD (pg/ml)	Reg (pg/ml)			
MCSE 0176	341,000	5,000	1,070	100	−	
	118,000	5,025	1,530	1	+	17
NOWR 1192	469,900	209	20	3,125	−	
	82,400	41	0	ND	+	1
EMJA 0809	625,900	187	226	25	−	
	75,500	195	216	ND	+	6
TIMI 0018	3,450,000	330	390	100	−	
	235,000	35	35	0	+	2
NAPH 0073	1,800,000	480	480	1,000	−	
	182,000	320	320	100	+	17
MCMI 0063	232,000	221	12	625	−	
	134,300	160	7	ND	+	6
JOJI 0070	1,606,000	2,460	885	100,000	−	
	40,800	263	58	10	+	20
SLMI 0843	84,900	0	0	0	−	Week 0†
	18,000	0	0	ND	+	Week 1
	33,500	0	0	ND	+	Week 2
	28,100	0	0	ND	+	Week 6
	72,700	0	0	0	−	Week 7
ARLA 0846	49,100	0	0	0	−	Week 0
	7,300	0	0	ND	+	Week 1
	6,500	0	0	ND	+	Week 2
	11,200	0	0	ND	+	Week 6
	58,400	0	0	0	−	Week 7
MIWI 1278	173,600	79	0	0	−	Week 0
	21,900	28	0	ND	+	Week 1
	10,900	24	0	ND	+	Week 2
	9,200	31	0	ND	+	Week 6
	136,300	47	0	25	−	Week 7

*Parameters as for Table 1 †For kinetic analysis of viral load by RNA QC-PCR over a 6-week period of treatment with AZT, patients were studied before initiation of treatment (week 0), after 1, 2, and 6 weeks of treatment, and 1 week after temporary discontinuation of treatment (week 7).

Table 7 <u>Estimated Percent Change in Surrogate Markers from Baseline by Treatment Group at Week 1 and Week 6</u>

	L-697,661 50 mg	L-697,661 300 mg	L-697,661 1,000 mg	Zidovudine 500 mg
CD4 Count				
Protocol A (355/64)*				
Week 1	-9.5% (-22.3,5.4)†	6.6% (-8.0,23.5)	8.4% (-6.2,25.2)	15.7% (-0.2,34.2)
Week 6	-14.2% (-27.5,1.6)	-3.7% (-18.4,13.7)	-8.3% (-22.3,8.2)	-3.6% (-18.3,13.8)
Protocol B (304/60)*				
Week 1	-15.3% (-30.1,2.6)	53.2%≠ (25.2,87.3)	29.0%§ (5.7,57.6)	4.8% (-14.6,28.6)
Week 6	-32.1%¶ (-46.9,-13.2)	6.7% (-17.3,37.6)	-2.7% (-24.6,25.5)	4.6% (-20.4,37.4)
P24 Antigen				
Protocol A&B (328/60)*				
Week 1	-19.2%Π (-34.2,-0.8)	-31.9%¶ (-46.3,-13.6)	-42.1≠ (-53.9,-27.1)	-24.5% (-44.4,2.5)
Week 6	-20.9% (-40.6,5.3)	-34.1%‡ (-53.0,-7.8)	-17.0% (-39.9,14.7)	-25.1% (-50.9,14.2)

* (Number of observations in model/Number of patients)

† Estimated mean change from baseline at week 1 and week 6 (and 95% confidence interval) from mixed effects modelling of ratio to baseline (see Methods). All statistical comparisons represent significance in relation to change from baseline (pretreatment) values. Significant changes from baseline were observed only for those values indicated by the symbols (listed below).

≠ $P < 0.001$

§ $P = 0.012$

¶ $P = 0.002$

Π $P = 0.042$

‡ $P = 0.015$

Table 8 **Resistance-associated Amino Acid Mutations and Virus Susceptibility in Treated Patients**

Therapy Group‡	Virus Isolate		L-697,661 IC_{90} (uM)*	L697,661-Resistance Associated Substitutions¤	Zidovudine IC_{90} (uM)*	Zidovudine Resistance Associated Substitutions¤
500 mg x 2	108	pre	400	none	800	none
		post	≥12,000	Y181C	400	none
	139	pre	200	none	100	K70R
		post	≥12,000	Y181C	200	K70R
	154	pre	400	none	400	K70R
		post	≥12,000	Y181C	200	K70R
	437	pre	50	none	1,500	M41L,T215Y/F
		post	≥12,000	Y181C	1,500	M41L,T215Y
	452	pre	50	none	3,000	M41L,T215Y
		post	≥12,000	Y181C	800	K70R
100 mg x3	112	pre	100	none	50	none
		post	≥12,000	Y181C	25	none
	115	pre	50	none	100	none
		post	≥6,000	K103N,Y181C	50	none
	120	pre	100	none	12	none
		post	200	none	25	none
	428	pre	100	none	25	none
		post	800	K103Q	25	none
	430	pre	100	none	100	none
		post	50	K103N,Y181C	500	none
25 mg x2	116	pre	25	none	≥3,000	M41L,T215Y
		post	6,000	Y181C	≥3,000	M41L,T215Y
	129	pre	400	none	100	K70R
		post	400	none	200	K70R
	421	pre	200	none	≥3,000	M41L,T215Y
		post	200	none	≥3,000	M41L,T215Y/C
	431	pre	100	none	3,000	M41I
		post	≥12,000	Y181C	3,000	M41L/T215Y
	442	pre	200	none	800	none
		post	400	none	800	none
	466	pre	400	K103R	≥3,000	D67N,K70R,K219Q
		post	400	K103R	≥3,000	D67N,K70R,K219Q

AZT	422	pre	200	none	≥3,000	M41L,D67N,T215Y
		post	400	none	≥3,000	M41L,D76N,T215Y
	451	pre	50	none	100	none
		post	100	none	200	none
	460	pre	800	none	≥3,000	M41L,D67N,T215Y
		post	800	none	≥3,000	M41L,D67N,T215Y

† See text for description.

* Virus isolate sensitivity assays were performed as described in Methods.

≠ Amino acid substitutions are reported using the single letter amino acid code. The number refers to the residue position within the reverse transcriptase protein. The first letter is the amino acid residue found in wild-type, sensitive reverse transcriptase proteins. The second letter is the resistance-associated substitution identified in treated patients and known from in vitro studies to confer resistance (10,11,24). "None" indicates that no deviations from the wild-type sequence were noted at these residues [positions 103 and 181 for L-697,661 (24), and positions 41,67,70,215 and 219 for zidovudine (10,11)].

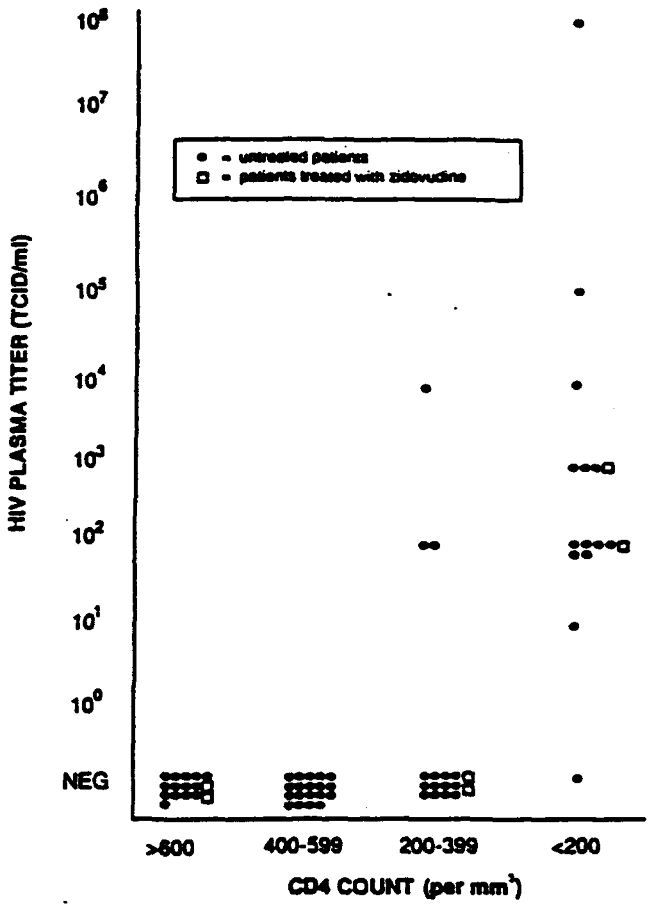


Figure 1. Plasma titers of human immunodeficiency virus (HIV) type 1 in adults. Magnitude of plasma viremia by CD4 counts (cells/mm^3).

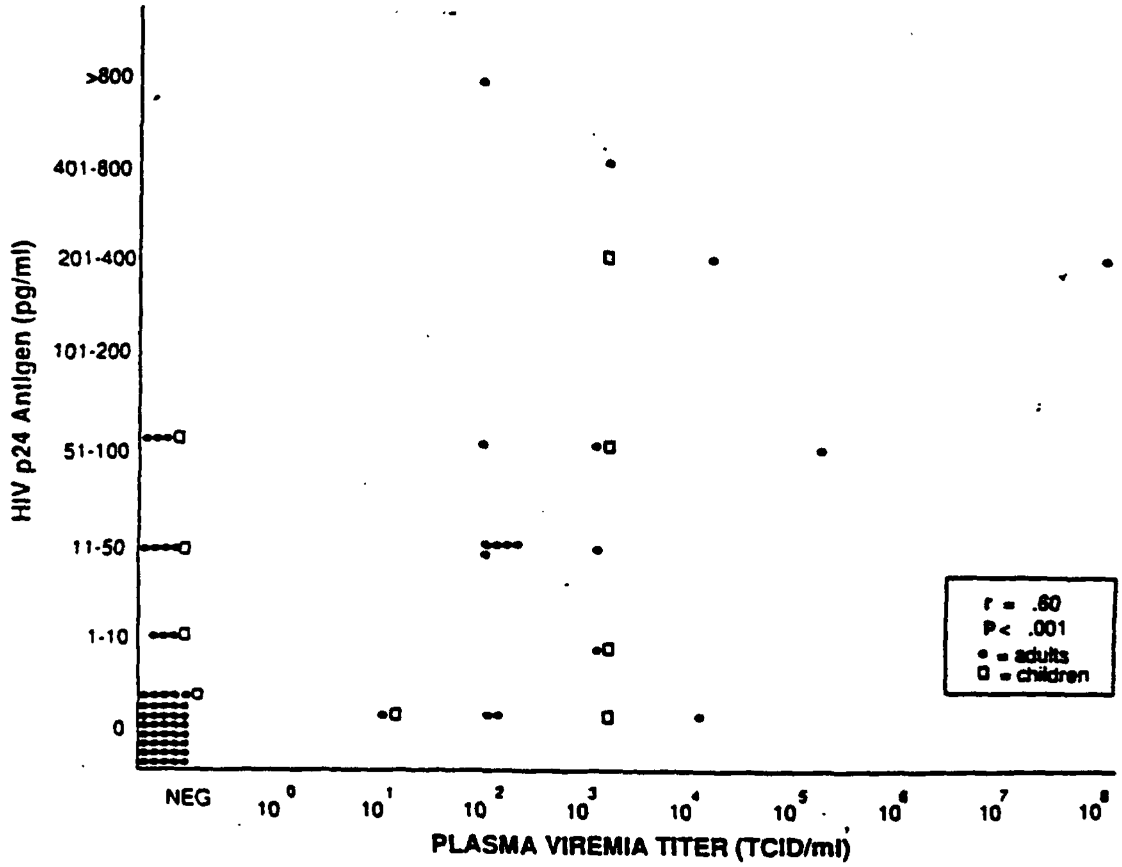
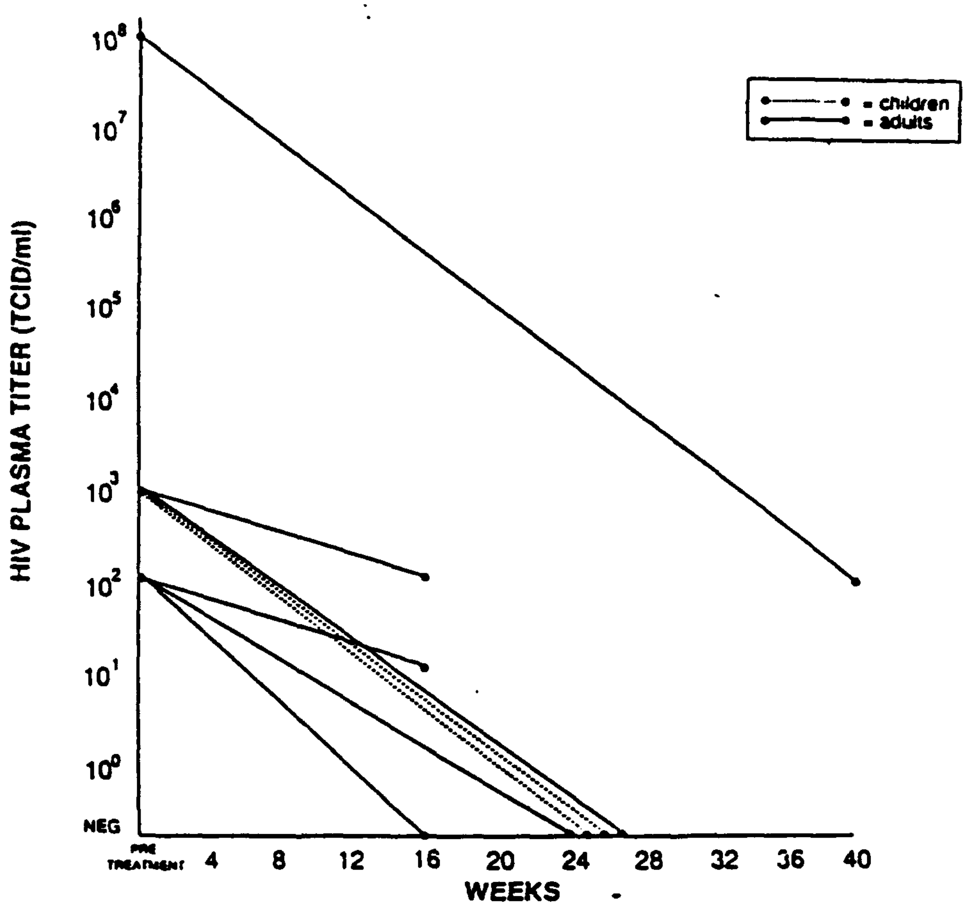


Figure 2. Comparative analyses of human immunodeficiency virus (HIV) type 1 plasma viremia titers and p24 antigen levels in adults and children. Neg, no growth.

Figure 3. Changes in human immunodeficiency virus (HIV) type 1 plasma viremia titers in adults and children before and after treatment with zidovudine. Neg, no growth.



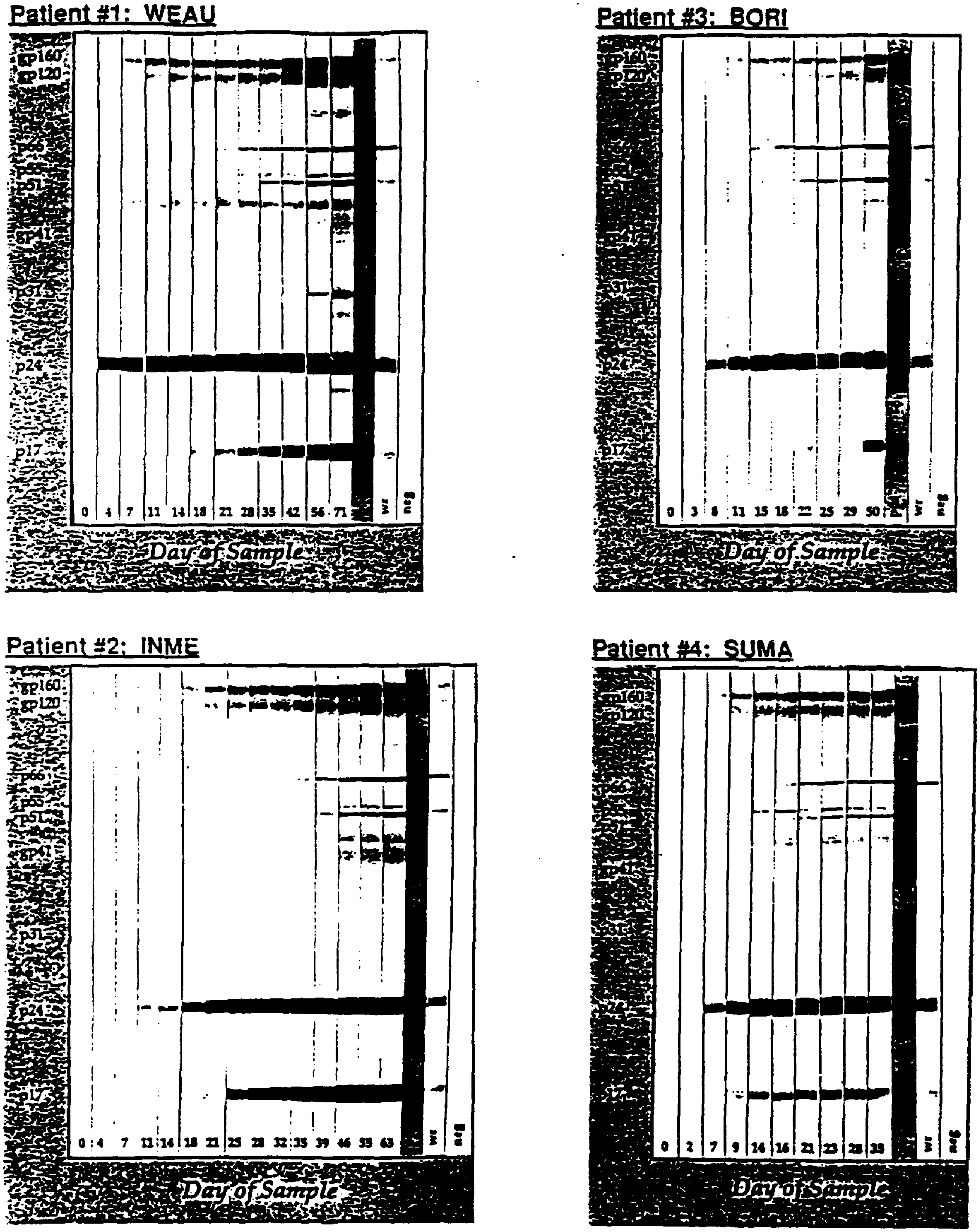
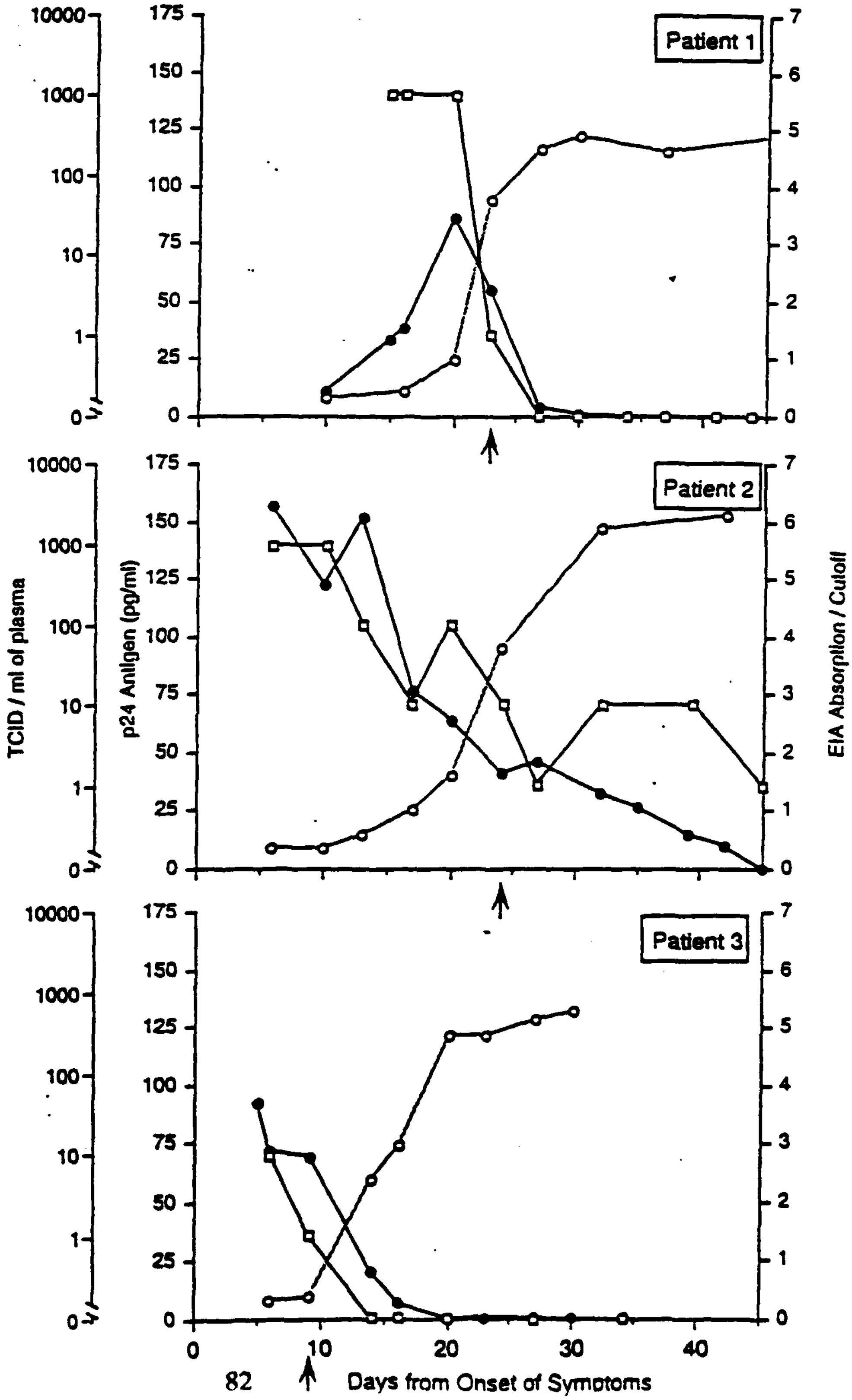


FIGURE 4: Seroconversion profiles for four subjects with acute HIV-1 infection resulting from sexual transmission.

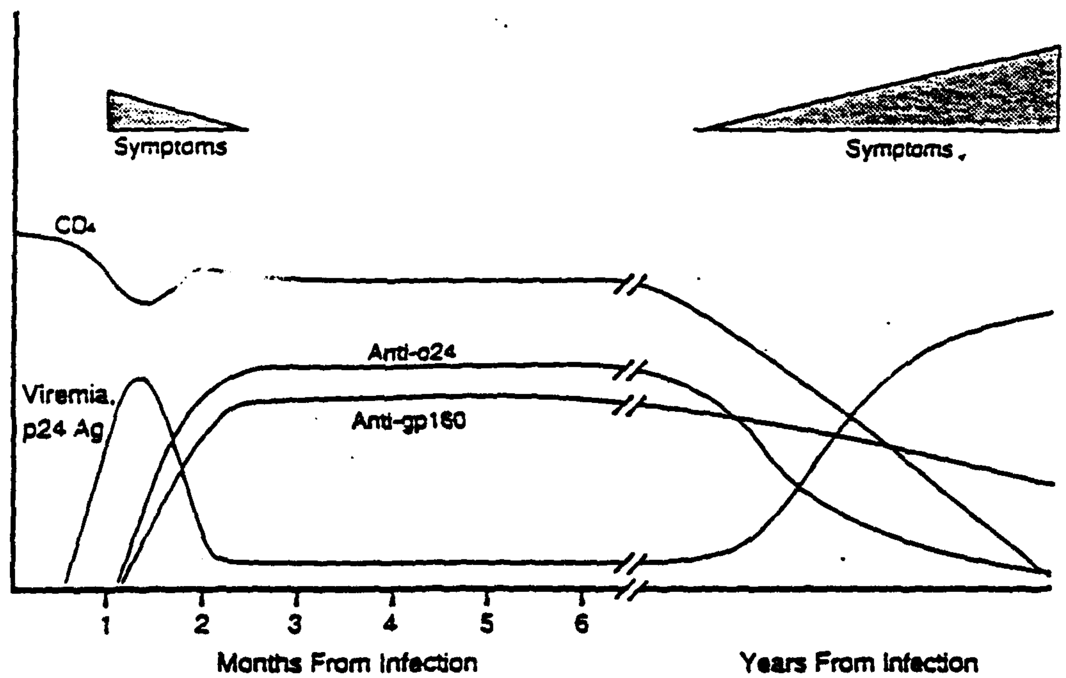
FIGURE 5: Plasma virus titers (squares) are expressed as tissue-culture-infective doses (TCID) per milliliter, p24 antigen titers (solid circles) as picograms per milliliter, and antibody titers (open circles) as ratios of ELISA absorbance to cutoff. Arrows indicate the time of first detection of both HIV-1 p24 and gp160 antibody by Western blot analysis.



Patient	Date	# of clones	
RIER (Donor)	3/18/90	9	CTRPNNNTRKKITLGPGRVLYTTGEIIGDIRRAHC
		7	---------RS--I----AF-R--Q----------
		1	----H----RS--I----AF-R--Q--------R-
		1	-------------I-------------------R-
		1	-------------VS--------------------
		1	--G--------------------------------
WEAU (Recipient)	5/30/90	24	CTRPNNNTRKKITLGPGRVLYTTGEIIGDIRRAHC
	6/21/90	17	-----------------------------------
		1	--------------------------M--------
		1	---------------------------------R-
		1	--------------------------V--------
	1/18/91	10	-----------------------------------
		11	-I---------------------------------
		1	-I--------------------I------------

FIGURE 6: Evolution of V3 envelope loop sequences associated with HIV-1 transmission and acute infection.

FIGURE 7: Natural history model of HIV-1 infection.



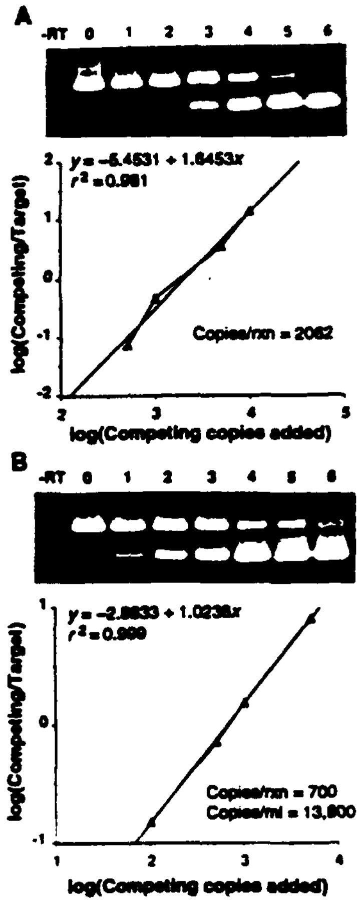


Fig. 8 QC-PCR quantitation of HIV-1 RNA. Video images are shown of electrophoretically resolved, ethidium bromide–stained PCR products derived from wild-type target sequence (upper band, 260 bp) and the competitive template (lower band, 180 bp), along with plots used to determine copy numbers of target sequence in the specimens (*13, 17*). (**A**) Reconstruction experiment with in vitro transcripts from plasmid (pQP1) containing full-length target sequence (260-bp product) and plasmid (pQP1Δ80) containing internally deleted competitive template (180-bp product). Actual copy number added, 2000 copies pQP1 per reaction. (**B**) Quantitation of virion-associated HIV-1 RNA from plasma of BECH 0171, CDC stage II (Table 1). Lanes are as follows: −*RT*, PCR amplification without reverse transcription (to rule out contaminating DNA); lanes 0 to 6, 0, 100, 500, 1,000, 5,000, 10,000, and 50,000 copies of competing pQP1Δ80 transcript per reaction, respectively. Note absence of signal in all −RT lanes and absence of 180-bp signal in all 0 lanes (no competitive template added).

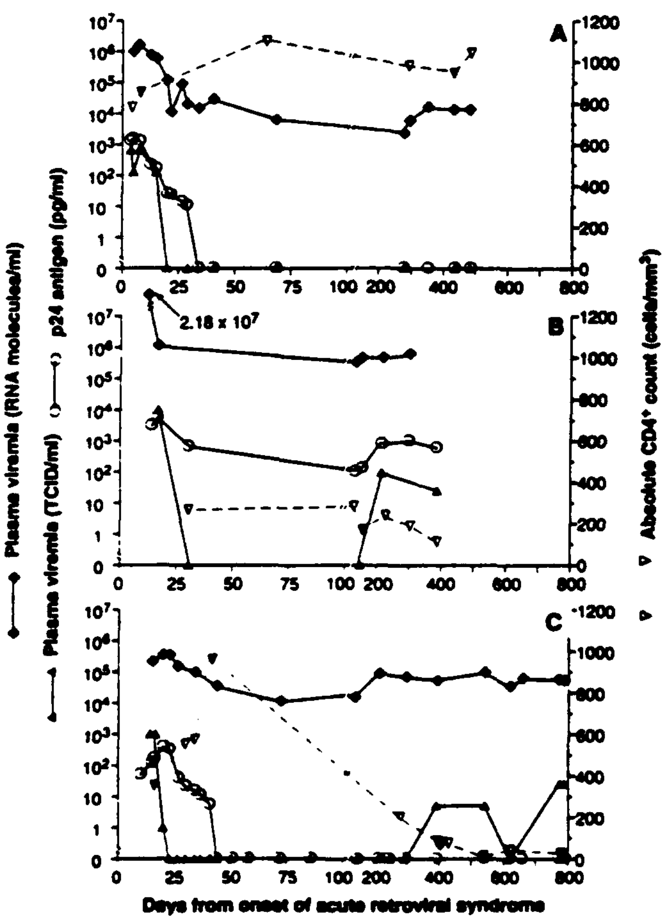
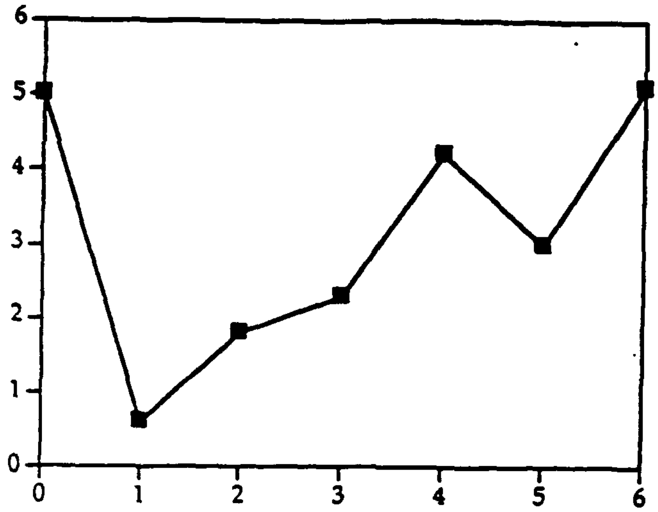


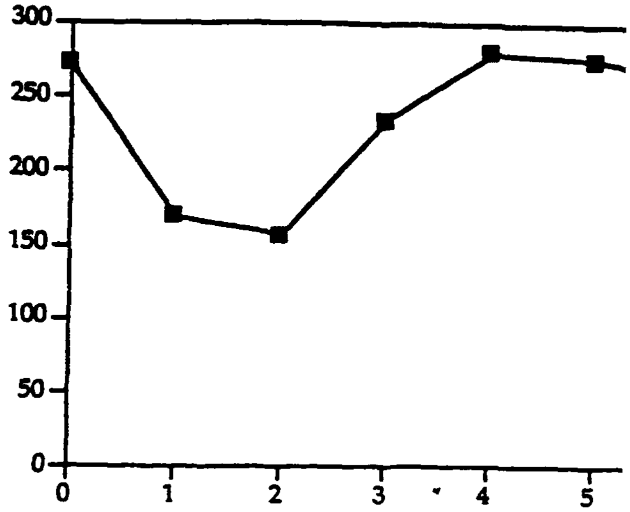
Fig. 9 Longitudinal determinations of HIV-1 RNA copy number by QC-PCR, p24 antigen levels by standard assay, culturable virus, and CD4⁺ T cell counts for patients presenting with symptomatic primary HIV-1 infection (CDC stage I). (**A**) Patient SUMA 0874. (**B**) Patient FASH 1057. (**C**) Patient WEAU 0575. Clinical history, partial virus culture results, and p24 antigen values [performed with a first-generation immunoassay kit (Abbott Laboratories, Chicago, Illinois)] for patient WEAU 0575 have been previously reported (7).

FIGURE LEGENDS

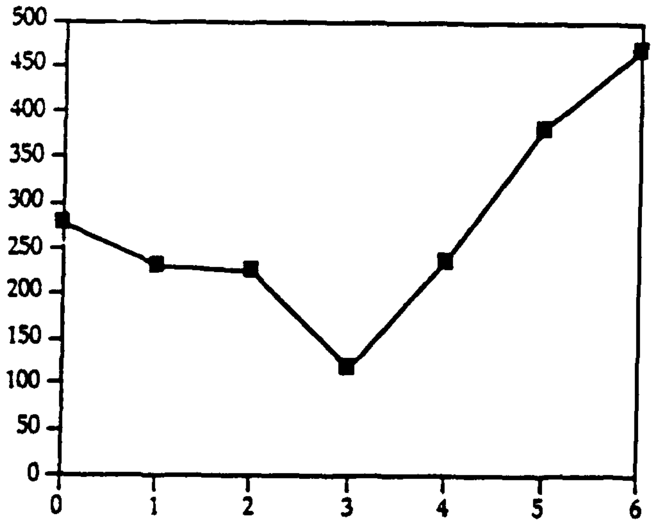
Fig 10 HIV-1 isolate sensitivity data and plasma virus p24 antigen levels from 8 representative patients. (A) Four patients treated with L-697,661 500 mg every 12 hours. (B) Four patients treated with L-697,661 100 mg every 8 hours. The plasma p24 antigen levels are plotted in pg/ml (y-axis) versus time of treatment in weeks (x-axis). Antigen levels were determined using a commercial p24 antigen assay (Coulter, Hialeah, FL) according to the manufacturer's directions. The IC_{90} values (nM) for inhibition of matched pre- and post-treatment virus isolates by L-697,661 and zidovudine are listed under each graph (see Methods section).



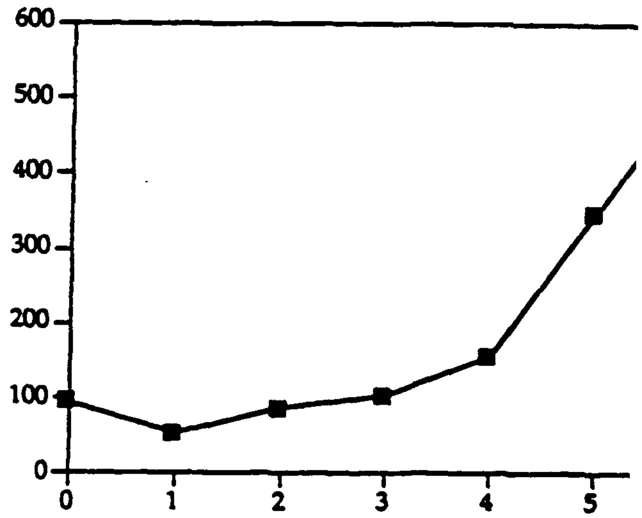
108	L-697,661	AZT
PRE	400	800
POST	≥12000	400



452	L-697,661	AZT
PRE	50	3000
POST	≥12000	800

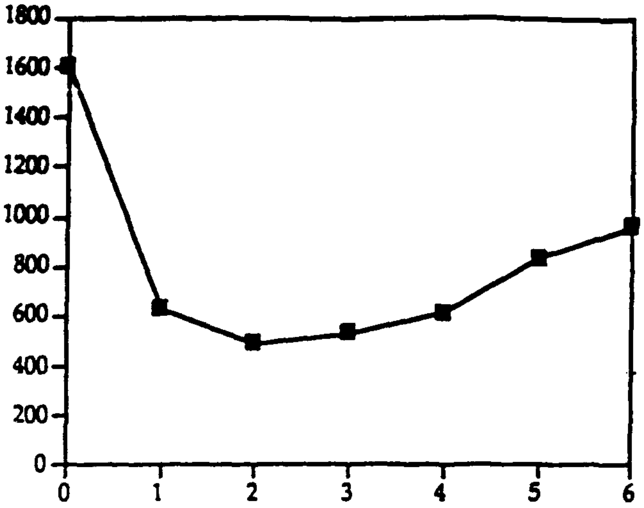


139	L-697,661	AZT
PRE	200	100
POST	≥12000	200

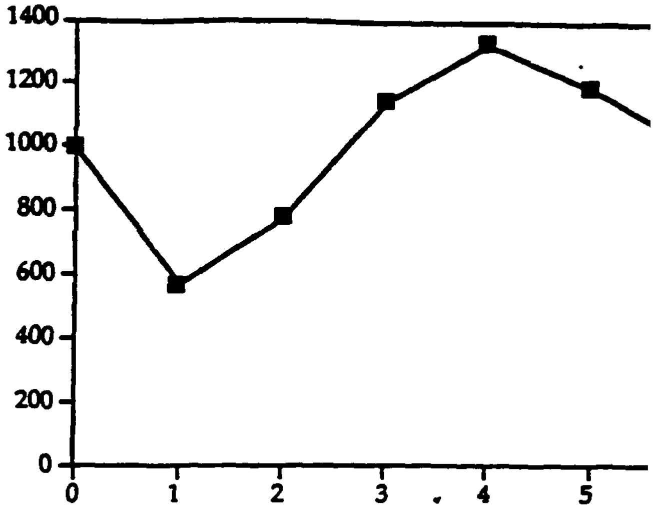


437	L-697,661	AZT
PRE	50	1500
POST	≥12000	1500

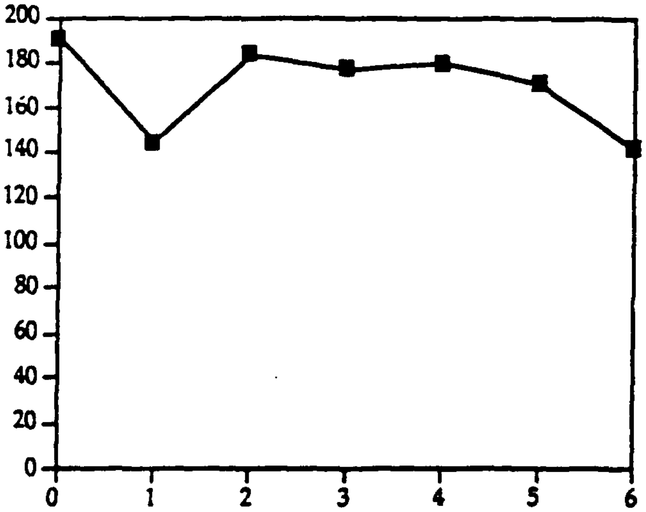
Fig 10



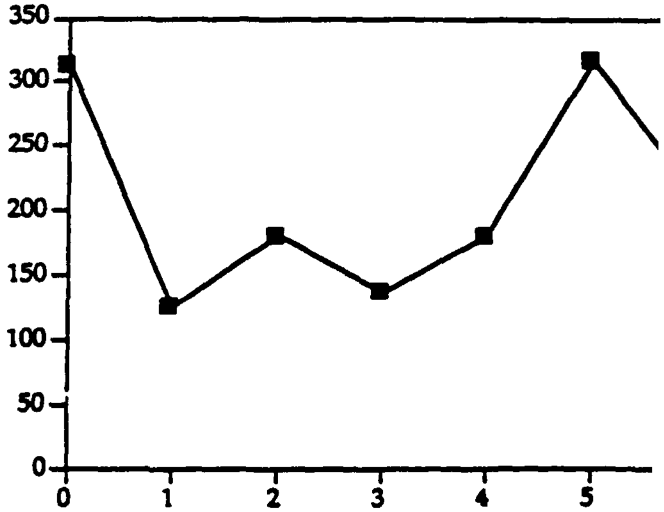
112	L-697,661	AZT
PRE	100	50
POST	≥12000	25



430	L-697,661	AZT
PRE	400	100
POST	≥12000	50



115	L-697,661	AZT
PRE	50	100
POST	≥6000	50



428	L-697,661	AZT
PRE	100	25
POST	800	25

Fig 10 (cont)

B

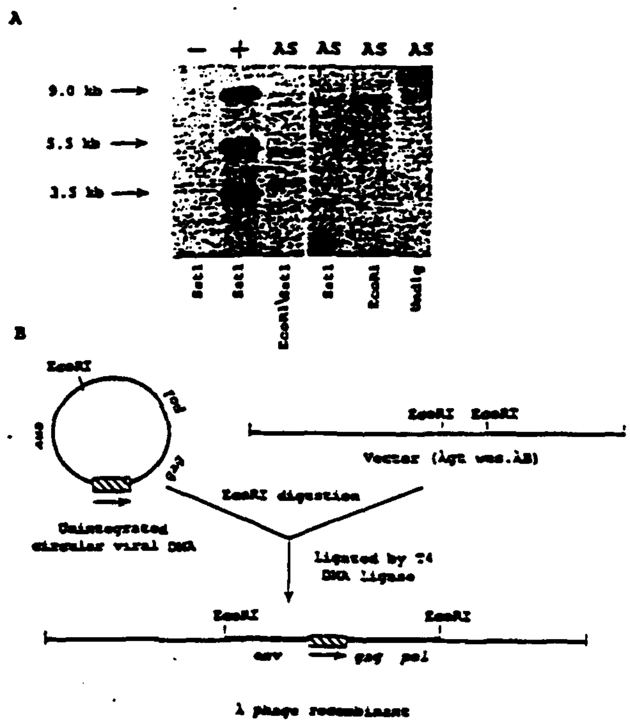


FIG. 11 Molecular cloning of full-length HIV-1 genomes from uncultured human brain. (A) Southern blot analysis of high-molecular-weight brain DNA derived from a patient with ADC. The restriction enzyme cleavage patterns and relative intensities of HIV-1 viral sequences in uncultured brain DNA (AS) are compared with those of positive (+; H9/HTLV-IIIb) and negative (−; uninfected PBMCs) control DNAs. Undig, undigested. (B) Schematic representation of the cloning strategy. Unintegrated circular viral DNA was linearized with *Eco*RI and subsequently cloned in permuted form into *Eco*RI-cleaved phage arms of λgtWES · λB.

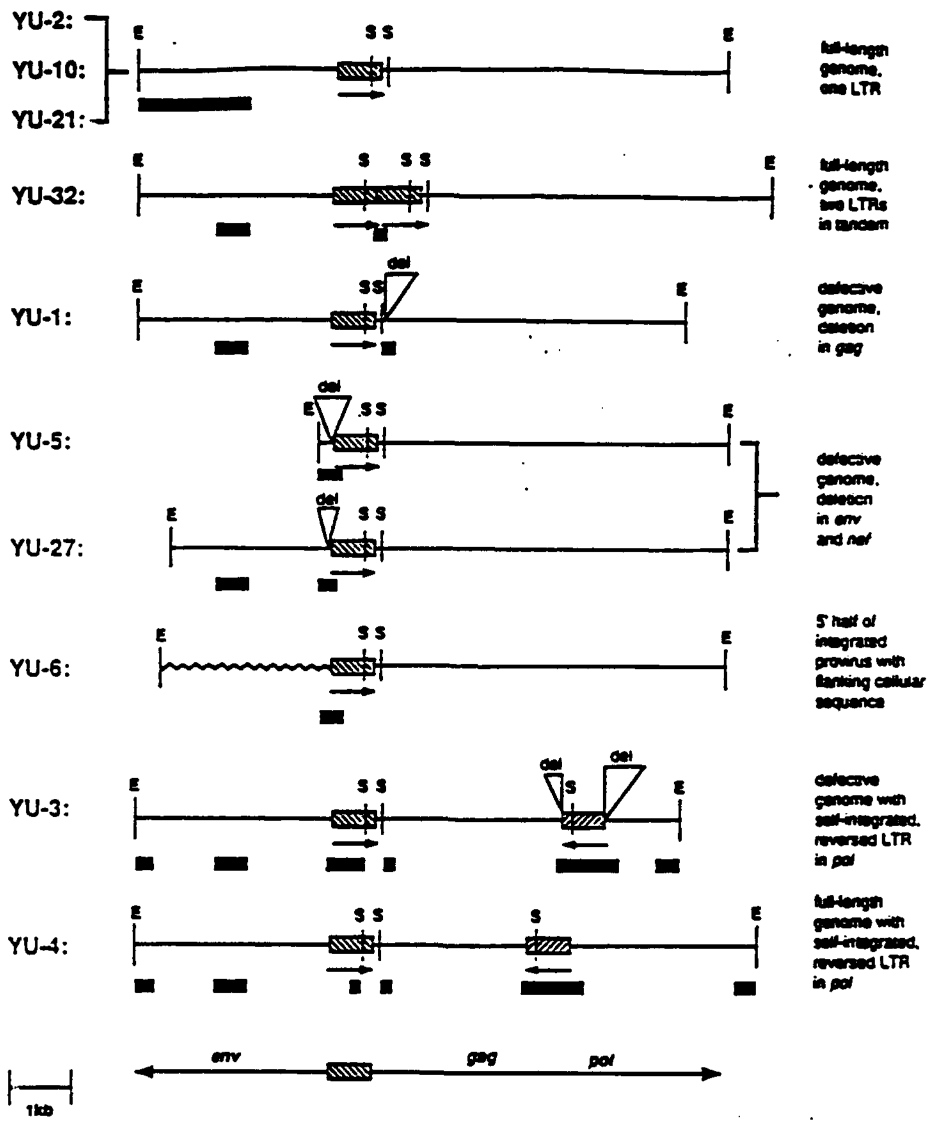


FIG. 12 Recombinant lambda phage clones obtained from a genomic library of uncultured brain DNA. Restriction enzyme analysis identified nine clones as containing unintegrated HIV-1 circles in permuted orientation and one clone as representing an integrated proviral half with flanking cellular sequences (wavy lines). Hatched boxes represent LTR sequences; arrows indicate the transcriptional direction. Triangles depict the positions of internal deletions. Locations of restriction enzymes sites (E, *EcoRI*; S, *SstI*) and relative positions of major HIV-1 open reading frames are shown. Genomic regions confirmed by nucleotide sequence analysis are shaded.

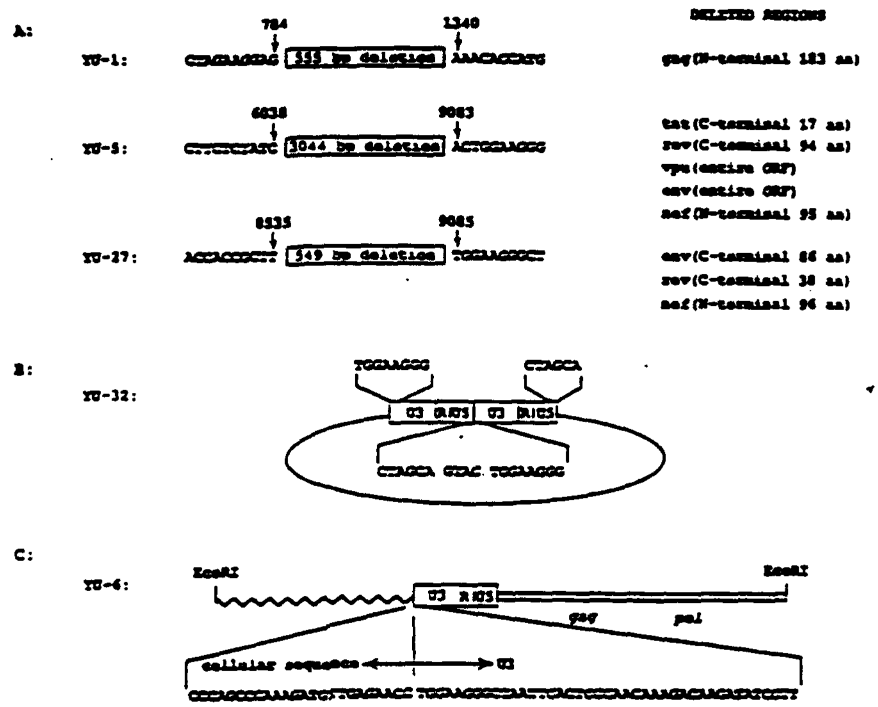


FIG. 13 Nucleotide sequence analysis of selected genomic regions in brain-derived HIV-1 genomes. (A) Molecular characterization of internal deletions. The extents and locations of internal deletions in clones YU-1, YU-5, and YU-27 are depicted. Sequences are numbered according to the HXB2 reference clone (36). (B) Schematic representation of the circle junction in clone YU-32. The orientation of both LTRs was confirmed by sequence analysis. An additional 4 bp located between the 3' terminus of the 5' LTR and the 5' terminus of the 3' LTR are shaded. (C) Sequence analysis of the junction between HIV-1 proviral DNA and flanking cellular sequences in clone YU-6. The boundaries of the integration site are depicted. aa, amino acids.

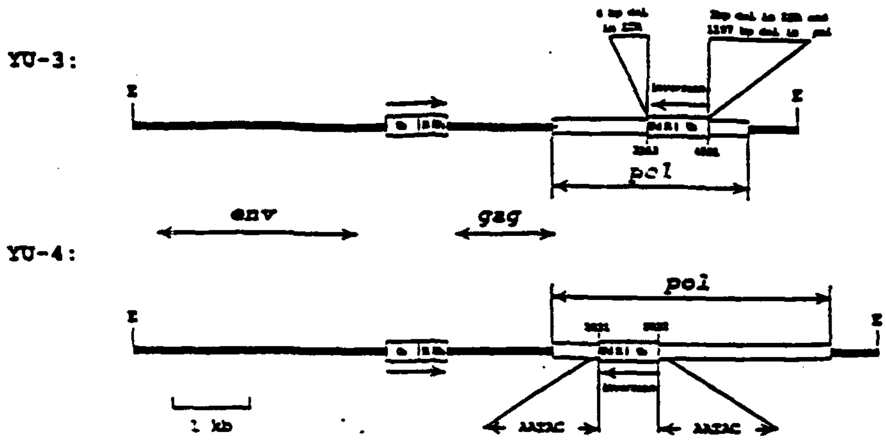


FIG. 14 Identification of inverted LTR sequences in the *pol* gene of two unintegrated HIV-1 circles. Nucleotide sequence analysis confirmed the boundaries of the insertion sites. YU-3 contains deletions in both inverted LTR and adjacent *pol* sequences. YU-4 is characterized by an intact inverted LTR flanked by a 5-bp direct repeat (AATAC) immediately adjacent to the insertion site. Sequences are numbered according to the HXB2 reference clone (36).

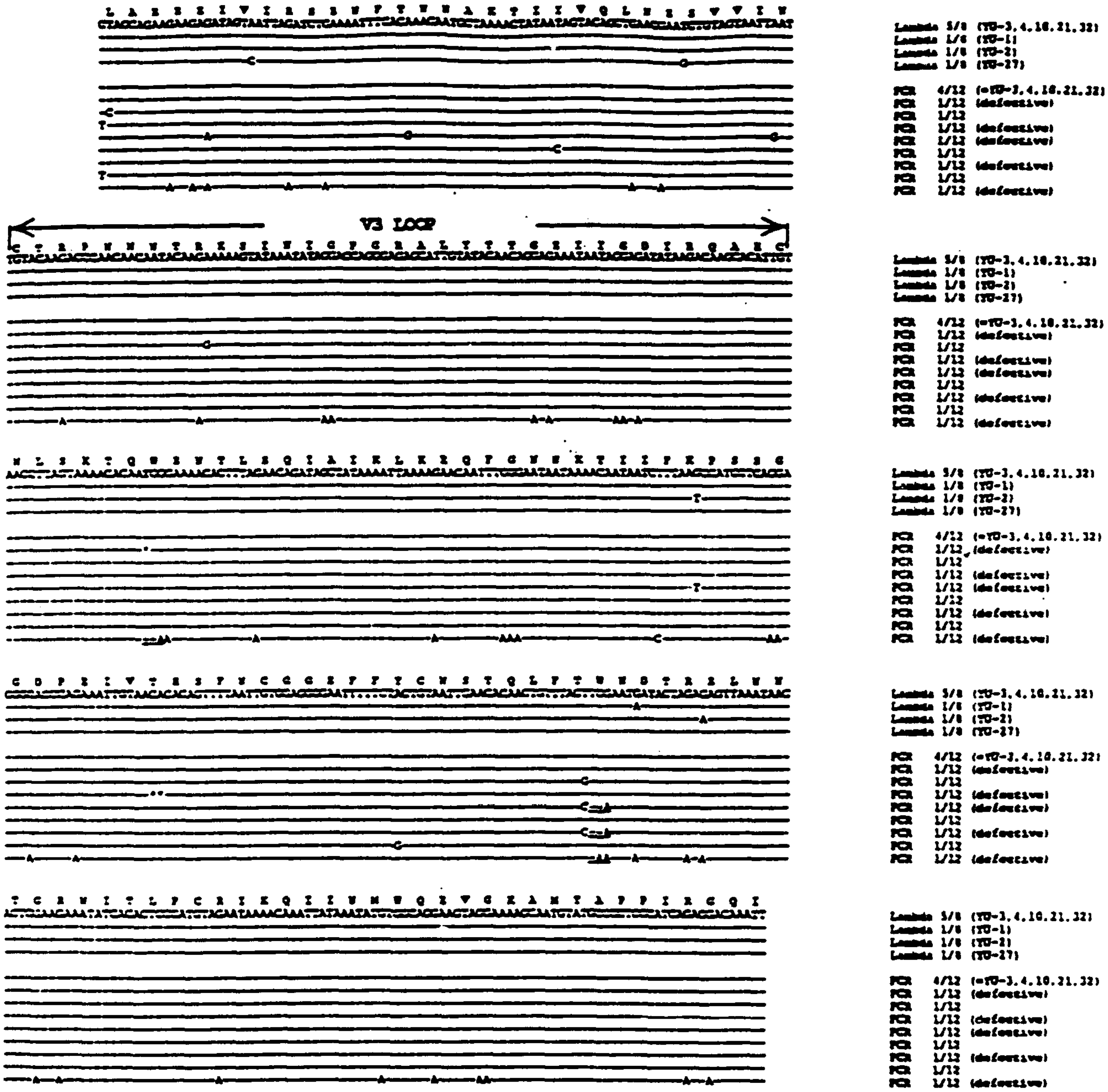


FIG. 15 Genetic variation of HIV-1 in primary human brain. A 510-bp stretch of envelope sequence is aligned between 8 lambda phage-derived and 12 PCR-derived clones. The deduced amino acid sequence, including the position of the major V3 neutralizing epitope, is shown above the nucleotide sequence. Sequence changes are depicted in reference to the predominant viral form, which represents five lambda phage-derived and four PCR-derived clones. Dashes indicate nucleotide sequence identity; asterisks represent single-base-pair deletions. Underlined nucleotides indicate the positions of in-frame translational stop codons. The frequencies of lambda phage- and PCR-derived clones with identical sequences along with the total number of clones analyzed are shown.

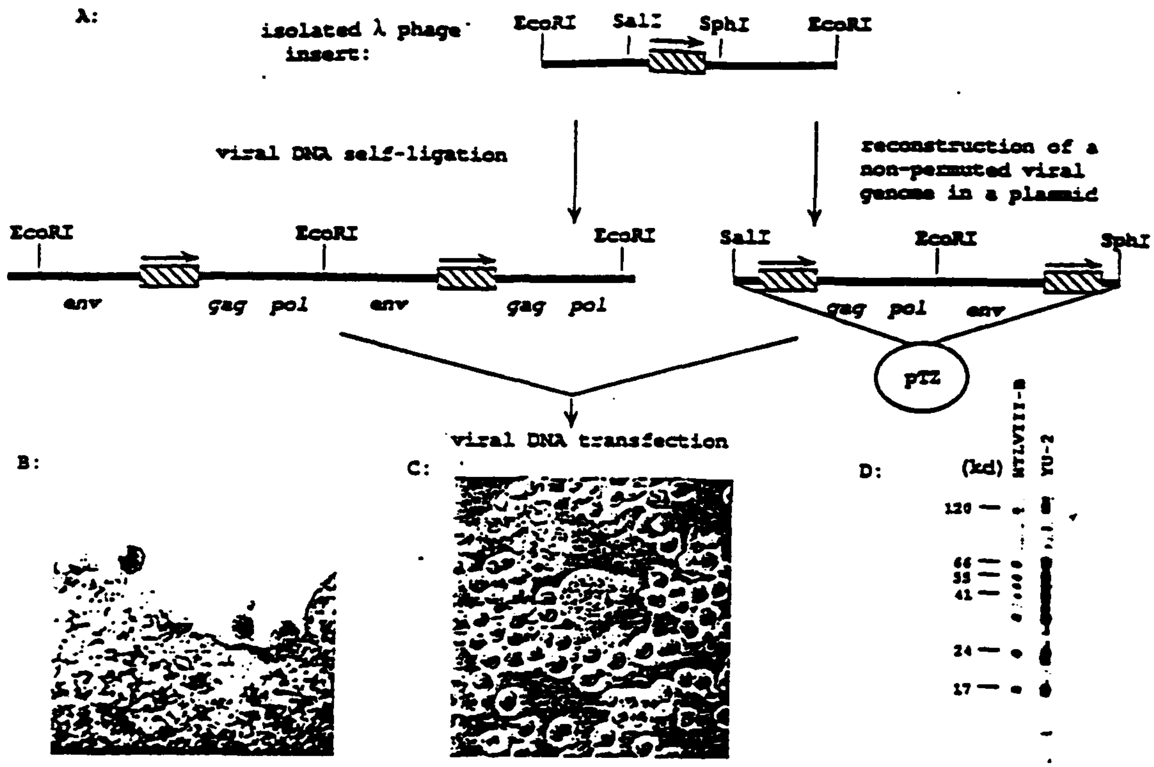


FIG. 16 Biological analysis of a replication-competent HIV-1 genome cloned directly from uncultured human brain. (A) Reconstruction of viral genomes in nonpermuted orientation. Lambda phage inserts were isolated following digestion with *EcoRI* and subsequently self-ligated. Correctly oriented viral inserts were also obtained by ligating a *SalI-EcoRI* fragment and an *EcoRI-SphI* fragment into the plasmid vector pTZ19R. (B) Electron micrograph of transfection derived YU-2 virus in normal donor PBMCs. (C) Virus-induced syncytium formation after cocultivation of YU2-infected PBMCs with Molt-4 (clone 8) cells. (D) Western blot analysis of cell-free YU-2 virus. All major viral gene products are compared with respect to presence and size with HIV-1 from H9/HTLV-IIIb-infected cells.

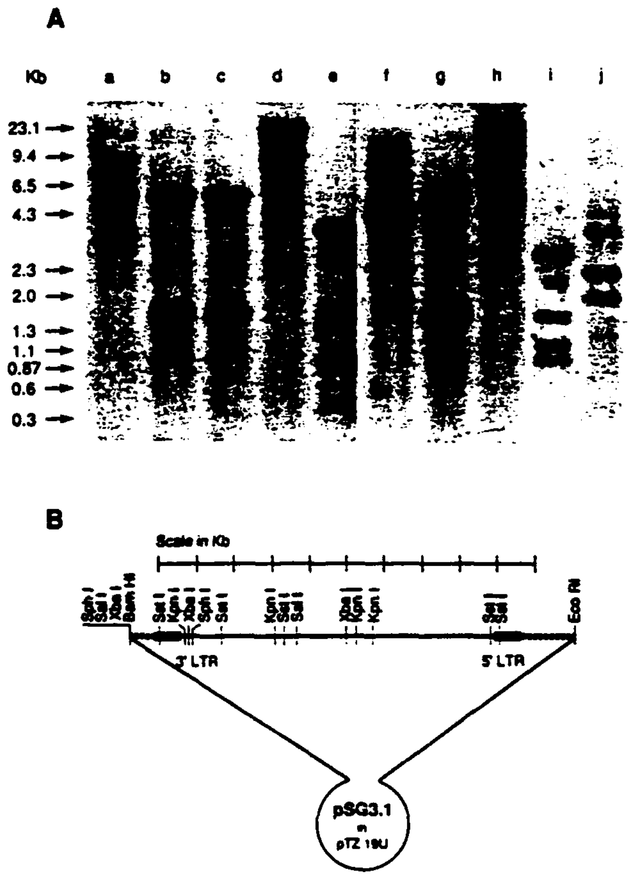


FIG 17 Restriction enzyme cleavage pattern of the HIV-1 isolate BC (A) and the replication competent proviral clone pSG3.1 derived from it (B). Southern blot analysis of genomic DNA from HIV-1_{BC}-infected Hut 78 cells (lanes b–j) was performed using a near full-length 8.9-kb *SstI-SstI* HIV-1 probe (pBH10i), as described (*50*). In lane a, an *SstI* digestion of the control virus strain HIV-1_{IIIB} revealed the expected fragments of 8.9, 5.5, and 3.4 kb, corresponding to proviruses polymorphic for an internal *SstI* restriction site (*50*). Lanes: b, *SstI*; c, *SstI/Bam*HI; d, *Bam*HI; e, *SstI/Xba*I; f, *Xba*I; g, *SstI/Eco*RI; h, *Eco*RI; i, *SstI/Kpn*I; j, *Kpn*I.

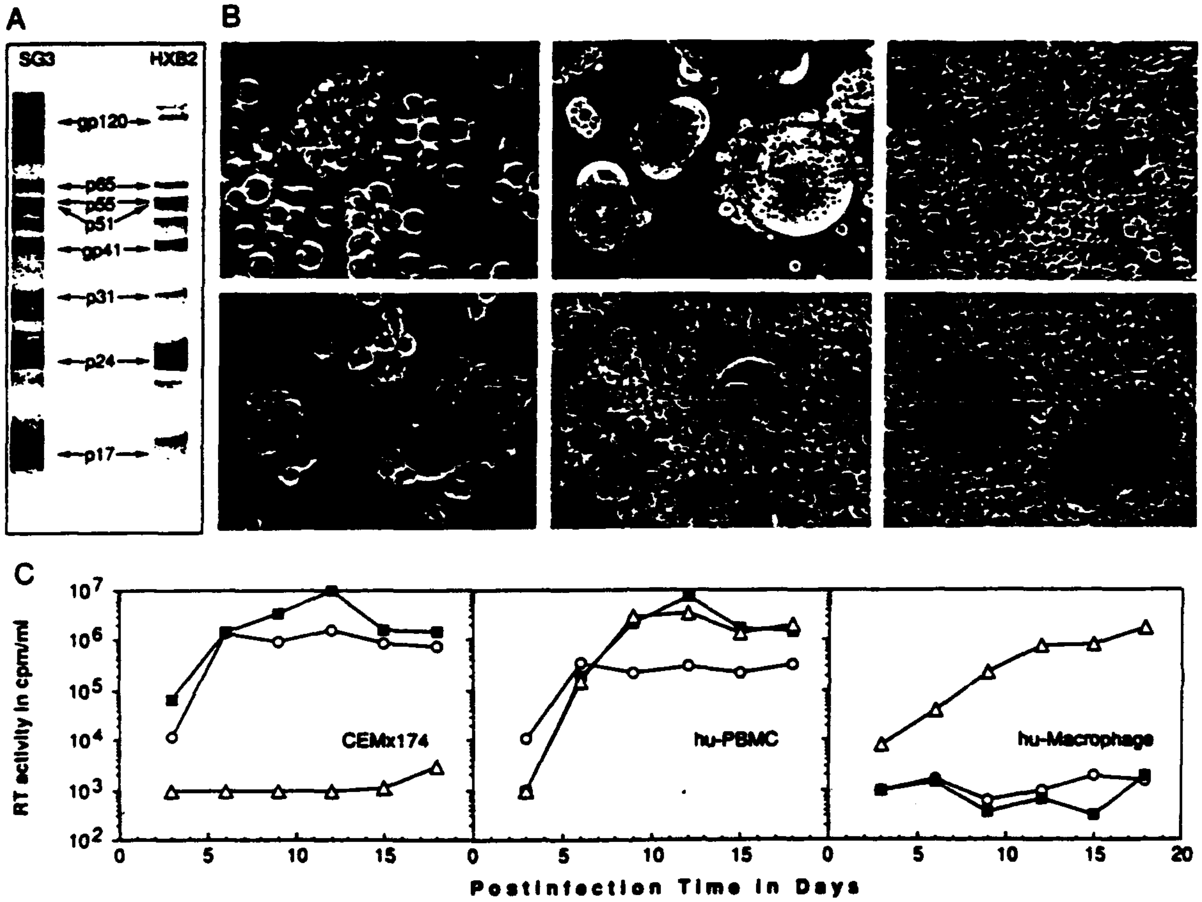


FIG 18 Viral protein composition (A), cytopathic activity (B), and replication characteristics (C) of SG3. Western immunoblot analysis (A) was performed using pelleted SG3 virions grown in CEMx174 cells and probed with serum from an HIV-1-infected human subject. A similar preparation of HXB2 virus was used as a reference. Syncytium induction was analyzed (panel B, clockwise from top left) in acutely infected human PBMCs as well as Sup T1, Jurkat, Molt 4 clone 8, CEMx174, and H9 T-cell lines. Replication (C) of SG3 (O) and two other reference viruses, HXB2 (■) (*11*) and the monocyte tropic clone YU2 (Δ) (*34, 35*), were assessed in CEMx174 cells, human PBMCs, and purified human monocyte-macrophage monolayers. Ten million PBMCs, or 1×10^6 CEMx174 cells, were infected with equal amounts of the respective viruses (2.5×10^5 cpm RT) derived by Cos-1 cell transfection of proviral DNA. Virus production was monitored by measuring supernatant RT activity as described (*34*). For monocyte-macrophage cultures, 0.5×10^6 adherent cells were infected with virus inocula equivalent to 7.5×10^4 cpm RT activity. The shaded area at the bottom of Figs. 2–4 represents the background range for the RT measurements.

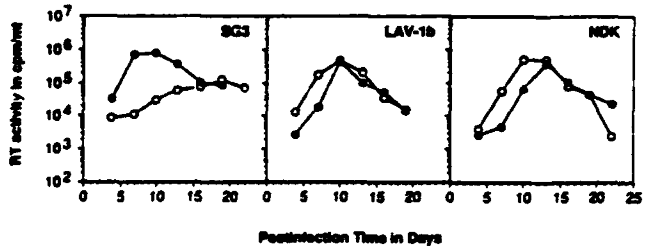


FIG 19 Replication of SG3, LAV-1b, and NDK in primary PHA-stimulated chimpanzee (●) and human (O) PBMCs. Ten million cells were infected with viral inocula of 1 × 10^4 $TCID_{50}$ titered on PHA-stimulated human PBMCs and were assayed periodically for virus production as described in the legend to Fig. 2.

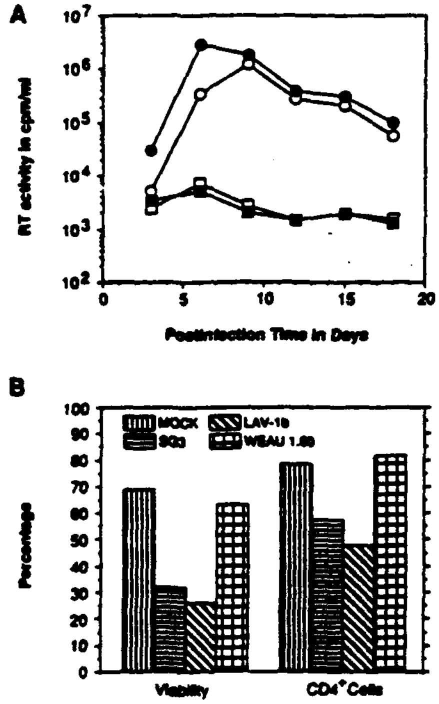


FIG 20 Replication (A) and cytopathicity (B) of SG3 (●), LAV-1b (○), WEAU 1.60 (□), and Mock (■) virus in CD4+-enriched chimpanzee lymphocytes. CD4+ lymphocytes were prepared by immunobead negative selection (Dynabeads, Dynal), according to the manufacturer's recommendations. Ten million cells were infected with viral supernatants containing 1×10^4 $TCID_{50}$ titered on human PBMCs, as described in the legend to Fig. 3. Cultures were assayed periodically for virus production (A), and after 12 days, for viability and CD4+ cell percentage (B).

A

B

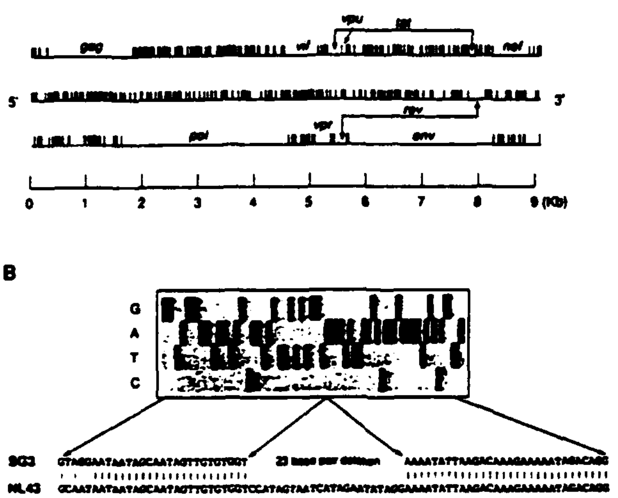


FIG 21 The complete nucleotide sequence of pSG3.1 was determined by dideoxy nucleotide sequencing, as described (34) and has been deposited with the GenBank Data Library under Accession No. L02317. The potential open reading frames (A) were determined using Microgenie (Beckman) and University of Wisconsin Genetic Computer Group software packages. A 23 base pair deletion was identified in the *vpu* gene of SG3 (B) and is shown in comparison with the complete *vpu* gene in HIV-1_{NL43} (42).

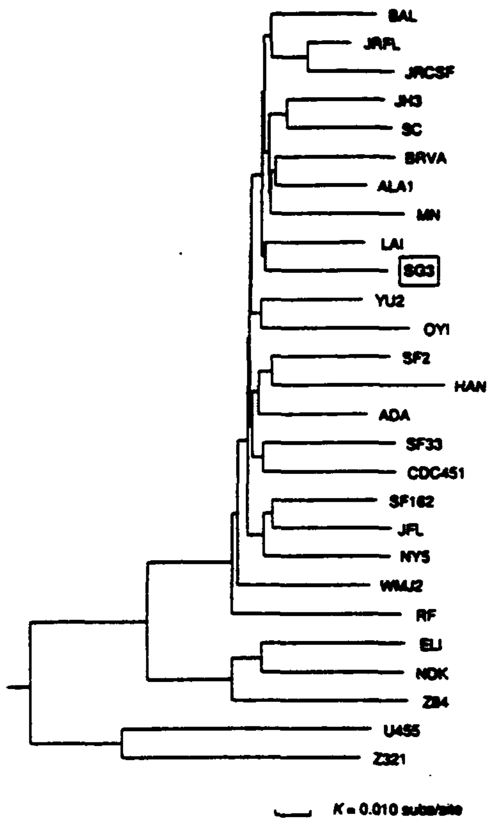


FIG 22 Phylogenetic relationship of SG3 to other HIV-1 isolates, derived by comparison of *env* gene sequences. The derivation of sequences is summarized in Refs. (*34*) and (*42*). Predicted protein sequences were aligned using the CLUSTAL program (*22, 23*). Three short regions of problematical alignment (see Ref. *42*) were excluded from further analysis so that an average of 806 codons was compared between sequences. Distances between the aligned gene sequences were then determined as a function of *K*, the estimated number of nucleotide substitutions per site, according to the method of Li *et al.* (*37*); *K* is the weighted average of the numbers of substitutions per synonymous and per nonsynonymous site, each corrected for multiple hits allowing for different rates of transitions and transversions. Phylogenetic relationships among the sequences were estimated by the neighbor-joining method (*46*) applied to a matrix of these pairwise distances; the tree was rooted by reference to SIV_{CPZ} as an outgroup. Horizontal branch lengths are drawn to scale (the bar represents *K* = 0.010 substitutions per site, or 1% divergence); vertical separation is for clarity only.

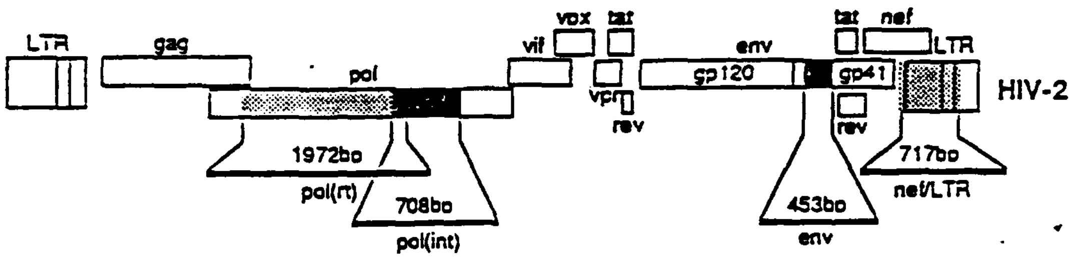


FIGURE 23 Location of HIV-2 sequences amplified from uncultured PBMC DNA. Dark shaded areas highlight *pol* (integrase; 708 bp) and *env* (453 bp) fragments amplified from all three study subjects (FO784, 2238, 7312A). Lighter shaded areas indicate *pol* (reverse transcriptase; 1972 bp) and *nef*/LTR (717 bp) fragments amplified only from subject FO784.

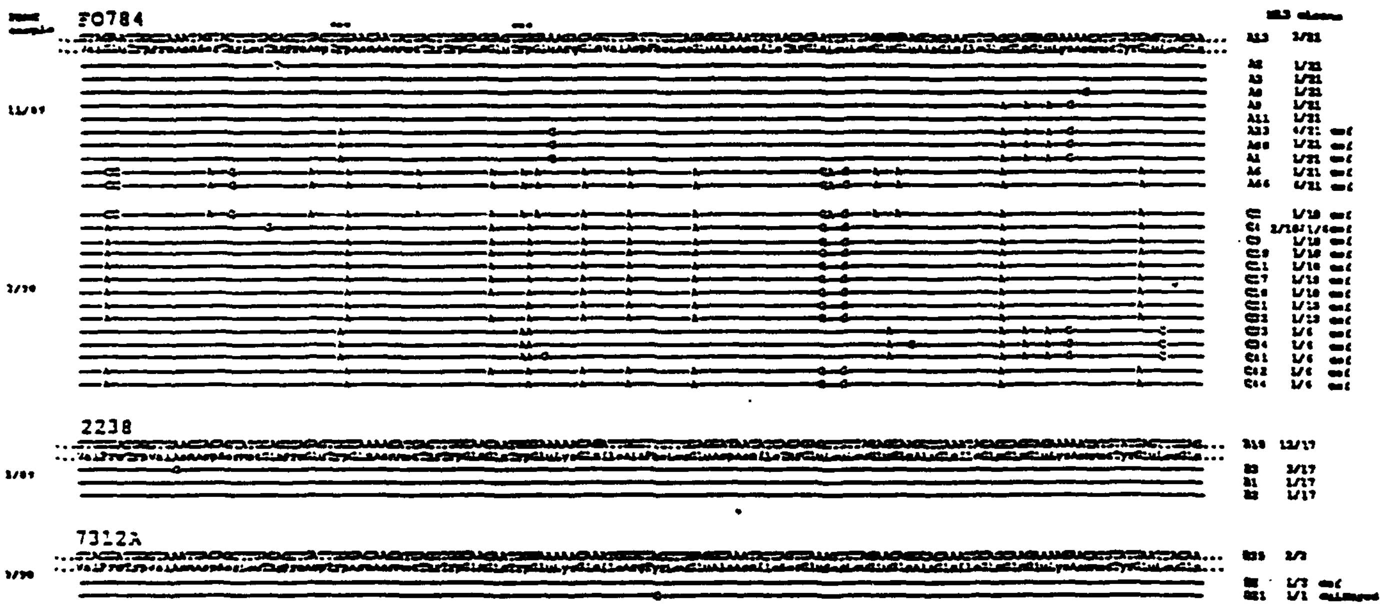


FIGURE 24 Intrastrain variability and G to A hypermutation in HIV-2. An alignment of 150 bp *env* sequences is shown. Nucleotide substitutions in this segment are representative of the extended *env* and *pol* sequences. Individual clones are compared to a predominant, non-truncated clone with dashes indicating sequence identity. The total number of recombinant M13 clones analyzed, the number of distinguishable clones per amplification reaction, and the number of defective clones (def) with premature truncations are indicated. Asterisks indicate premature stop codons resulting from G to A hypermutation.

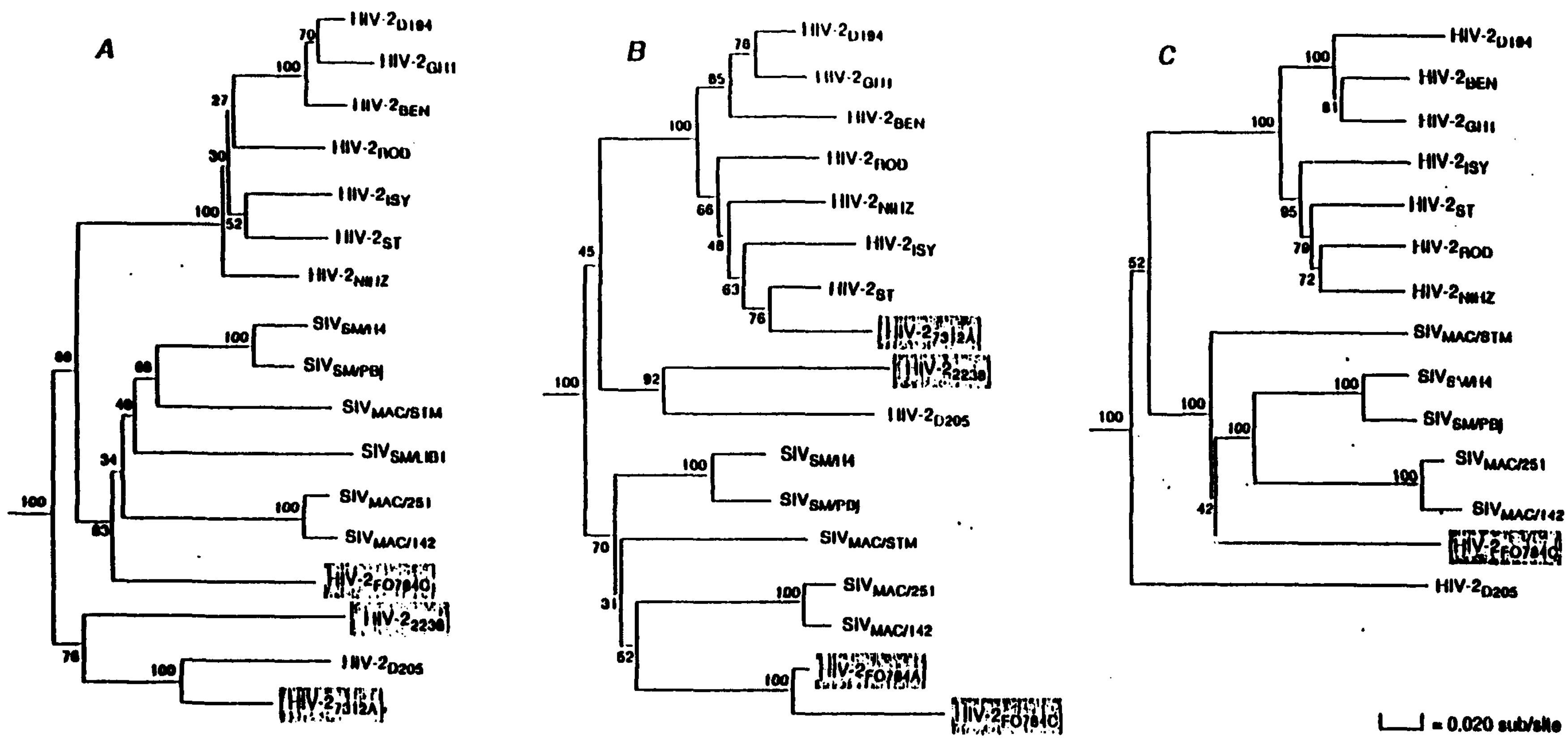


FIGURE 25 Phylogenetic relationships among (A) *pol*-integrase sequences, (B) *env* sequences, and (C) *pol*-reverse transcriptase sequences from HIV-2_{FO784}, HIV-2_{2238}, and HIV-2_{7312A} (highlighted) and other HIV-2, SIV_{SM}, and SIV_{MAC} isolates (56). Horizontal branch lengths are to scale; vertical separation is for clarity only. Numbers at each node indicate the percentage of bootstrap resamples (out of 5000) in which the cluster to the right is supported. The trees are rooted using HIV-1, SIV_{AGM}, and SIV_{MND} sequences (not shown); the branch lengths from the nearest node to the roots of the trees shown are 0.08, 0.11, and 0.11 substitutions per site for the *pol*-integrase, *env*, and *pol*-RT trees, respectively.

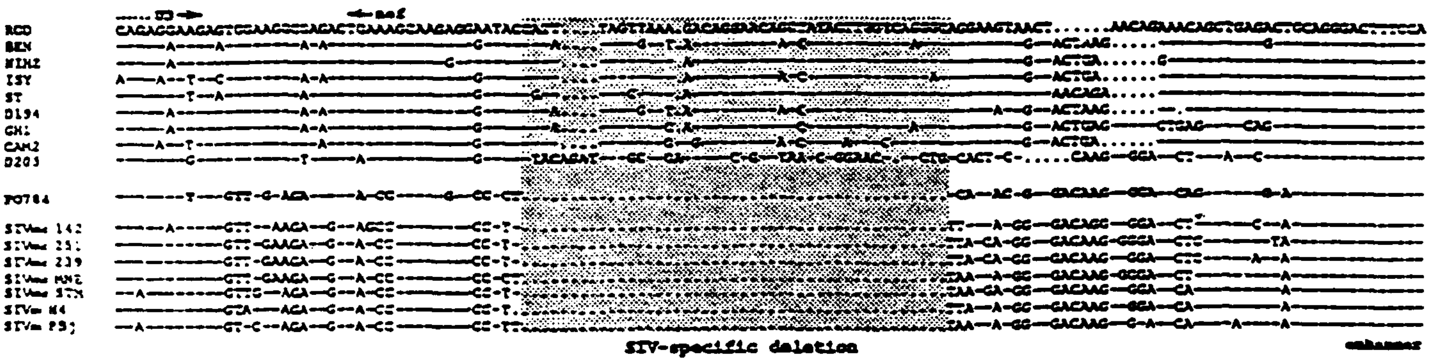


FIGURE 26 Identification of an SIV_{SM}/SIV_{MAC} "signature" sequence in HIV-2_{FO784}. HIV-2_{FO784} LTR sequences are aligned with prototype HIV-2 and SIV sequences in a region of the LTR encompassing a specific 40-44 bp insertion that is present in all previously reported HIV-2 viruses but absent in all SIV_{SM}/SIV_{MAC} viruses (56). Sequences are compared to HIV-2_{ROD}(46) as a reference sequence with dashes (-) indicating sequence identity and periods (.) indicating gaps introduced for optimal alignment. Enhancer sequences and the *nef* termination codon are indicated. HIV-2_{FO784} (boldfaced), like viruses of monkey derivation, lacked the 40-44bp insertion.

APPENDIX B: PUBLICATIONS BASED ON DAMD 17-90-C-0064

PUBLICATIONS

1. Clark, S.J., M.S. Saag, W.D. Decker, S.H. Campbell, J.L. Roberson, J.C. Kappes, P.J. Veldkamp, B.H. Hahn, and G.M. **SHAW**: High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection, New England Journal of Medicine 324:954-960, 1991.

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4. Li, Y., J.C. Kappes, J.A. Conway, R.W. Price, G.M. **SHAW**, and B.H. Hahn: Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. Journal of Virology 65:3973-3985, 1991.

5. Gao, F., L. Yue, M. Mixon, W.D. Decker, A.T. White, P.G. Pappas, B.M. Greene, G.M. **SHAW**, and B.H. Hahn: Biologically-unique, SIV-like HIV-2 variants in healthy West African individuals. In M. Girard and L. Valette (ed.) Retroviruses of Human AIDS and Related Animal Diseases, 5th Colloque des Cent Gardes. Paris, France, pp. 31-38, 1991.

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10. **SHAW, G.M.**: New insights into HIV-1 replication and pathogenesis. In M. Girard and L. Valette (ed.) Retroviruses of Human AIDS and Related Animal Diseases, Septieme

Colloque des Cent Gardes. Paris, France, pp. 11-15, 1992.

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12. Ghosh, S.K., P.N. Fultz, E. Keddie, M.S. Saag, P.M. Sharp, B.H. Hahn, and **G.M. SHAW**: A molecular clone of HIV-1 tropic and cytopathic for human and chimpanzee lymphocytes. Virology, 194:858-864, 1993.

13. Piatak, Jr., M., M.S. Saag, L.C. Yang, S.J. Clark, J.C. Kappes, K-C Luk, B.H. Hahn, **G. M. SHAW**, and J.D. Lifson: Determination of plasma viral load in HIV-1 infection by quantitative competitive polymerase chain reaction (QC-PCR). AIDS, in press.

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17. Graziosi, C., G. Pantaleo, L. Butini, J.F. Demarest, M.S. Saag, **G.M. SHAW**, and A.S. Fauci: Kinetics of HIV DNA and RNA synthesis during primary HIV-1 infection. Proceedings of the National Academy of Sciences, in press.

ABSTRACTS

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2. Li, Y., H. Hui, J.C. Kappes, R.W. Price, B.H. Hahn, and **G.M. SHAW**: Molecular cloning and analysis of replication competent HIV-1 proviruses from uncultured human brain in AIDS dementia complex. Keystone Symposia on Molecular Biology of Human Pathogenic Viruses, Lake Tahoe, California, 1991.

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SHAW, and B.H. Hahn: Identification of biologically unique, SIV-like HIV-2 variants in healthy West African individuals. HIV Disease: Pathogenesis and Therapy, Orlando, Florida, 1991.

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6. Clark, S.J., M.S. Saag, G. Pan, W.D. Decker, B.H. Hahn, and G.M. SHAW: Biologic and genetic analysis of human immunodeficiency virus type-1 associated with viral transmission and primary infection. ICAAC, Chicago, Illinois, 1991.

7. Gao, F., L. Yue, M.L. Mixon, W.D. Decker, A.T. White, P.G. Pappas, B.M. Greene, G.M. SHAW, and B.H. Hahn: Genetically and biologically unique strains of HIV-2. Annual Meeting, Laboratory for Tumor Cell Biology, Bethesda, Maryland, 1991.

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GRADUATE DEGREES: Yuexia Li, Ph.D.

APPENDIX C: PERSONNEL EMPLOYED ON CONTRACT DAMD 17-90-C-0064

Name/Degree	Position on Contract	Present Position
George M. Shaw, M.D., Ph.D.	Principal Investigator	Principal Investigator
Beatrice H. Hahn, M.D.	Co-Investigator	Co-Investigator
Sajal Ghosh, Ph.D.	Postdoctoral Fellow	Postdoctoral Fellow
Maria Taylor, M.S.	Laboratory Supervisor	Laboratory Supervisor
Sherri Campbell-Hill	Research Assistant	Research Assistant
Kelli T. Agnew	Laboratory Administrative Assistant	Laboratory Administrative Assistant
Ling Yue, M.D.	Research Assistant	Transfer
George Z. Pan, M.D.	Research Associate	Transfer
William D. Decker, B.S.	Research Assistant	Transfer
Joe Roberson, B.S.	Research Assistant	Transfer
Paul Chopra, B.S.	Research Assistant	Transfer
Yong Wu, M.D.	Research Assistant	Transfer